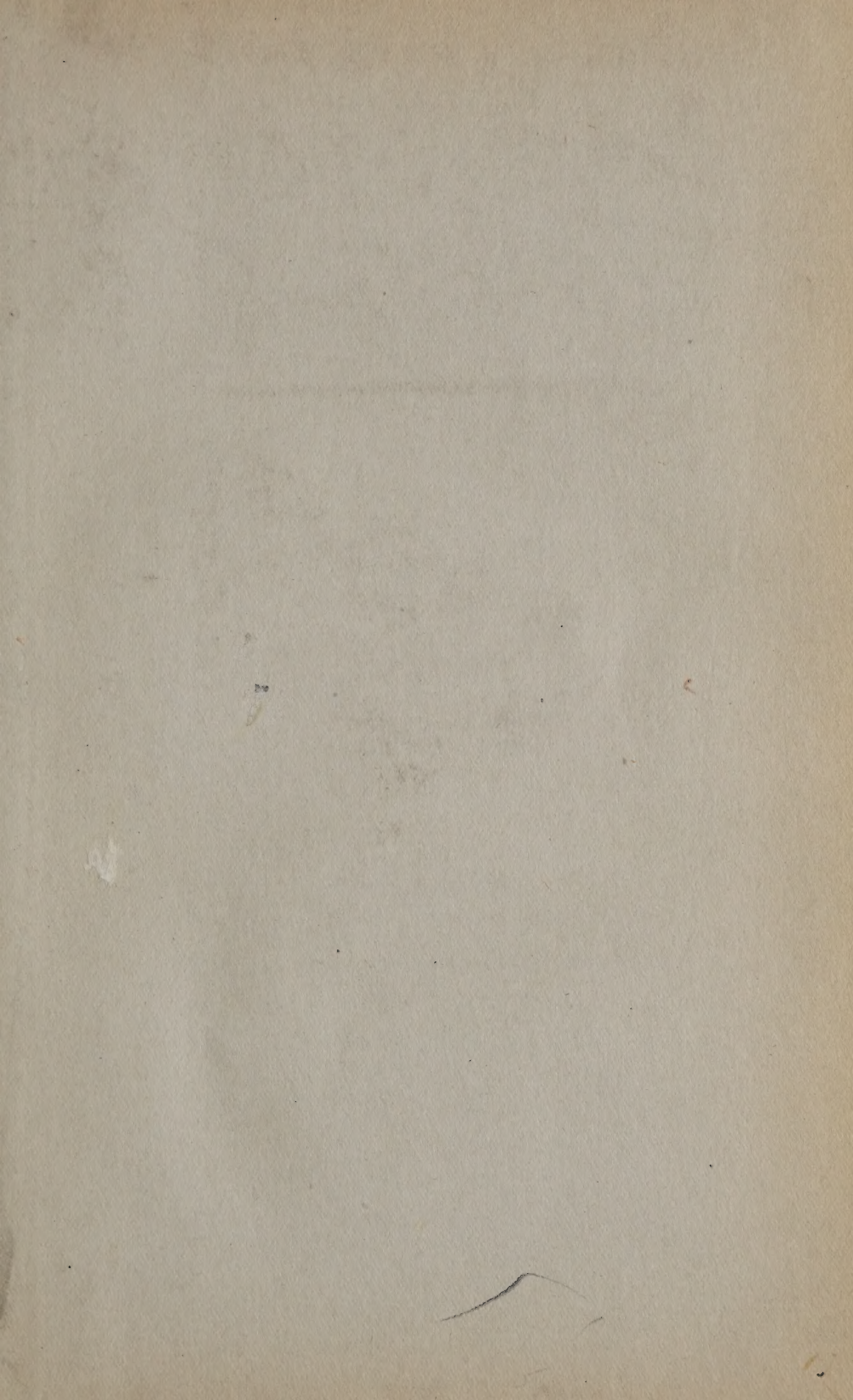




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CORRECTIONS.

On page 100, Vol. lix, No. 1, February, 1924, Salt mixture 185, for *Ca lactate 13.0* read *Ca lactate 130.0*.

On page 692, Vol. lix, No. 3, April, 1924, Table III, for the italic headings under "Muscle phosphates" read *mg. P per 10 gm.* instead of *mg. P per 100 gm.*

A METHOD FOR THE FRACTIONAL ANALYSIS OF INCOMPLETE PROTEIN HYDROLYSATES.

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(Received for publication, June 6, 1924.)

The constituents of an enzymatic hydrolysate of protein can be divided according to their complexity into six fractions; protein, metaprotein, proteose, peptone, subpeptones, and amino acids. A method for the quantitative estimation of these fractions was devised in order to secure more definite information regarding the changes occurring during hydrolysis than is obtained by the usual free amino nitrogen determinations. The method has stood the test of continued use by different workers for over a year, and has given consistently accurate results.

Briefly, the method involves the precipitation of the protein by trichloroacetic acid, of the metaprotein by careful adjustment of the reaction, of the proteoses by Na_2SO_4 at 33°C ., of the peptones by tannic acid under definitely fixed conditions; and the determination of the residual amino acids and simple peptides by a slight modification of the alcohol precipitation methods of Folin and Denis (1) and Van Slyke and Meyer (2). No new principle is introduced, but quantitative results with these reagents were found to be obtainable only under rigidly fixed conditions. The metaprotein fraction is determined on a separate aliquot of the original solution. For the assay of the other five fractions 100 cc. are required, containing not less than 200 mg. of nitrogen. The amount of each fraction is estimated by the nitrogen content, obtained by difference before and after filtration.

I.

Method of Fractional Analysis of Hydrolysate.

The total nitrogen of the hydrolysate is first determined on 5 cc. samples in triplicate. Then, to 40 cc. are added 10 cc. of

10 per cent trichloroacetic acid, and the mixture is allowed to stand for 1 hour. The solution is filtered and the total nitrogen determined on two 5 cc. portions of the filtrate. The difference between the first and second total nitrogen estimations is the sum of the protein and metaprotein.

To estimate the amount of metaprotein, a portion of the original solution of which the total nitrogen is known is brought to pH 6.0. At this hydrogen ion concentration the insolubility of albumin acid metaprotein was found to be greatest. The solution is filtered and the total nitrogen of the filtrate determined. After allowing for dilution, the difference between the total nitrogen of the original solution and of this filtrate is taken as a measure of the metaprotein content. The difference between the metaprotein nitrogen and the total trichloroacetic acid precipitable nitrogen is the protein nitrogen.

The remainder of the filtrate from the trichloroacetic acid precipitate, approximately 35 cc., is poured into a 35 cc. volumetric flask, provided with a long, wide neck, and graduations above and below the 35 cc. mark. It is then boiled in a water bath for 3 hours to decompose the trichloroacetic acid, and to drive off the resulting carbon dioxide and chloroform, cooled to room temperature, and made up to the original volume before boiling. Approximately 30 cc. are poured into a 50 cc. Erlenmeyer flask and 20 gm. of Na_2SO_4 are added. The excess of salt is advisedly as little as possible, in order to minimize the possibility of error due to abstraction of water by the hydrated crystals. The mixture of salt and solution is kept at 33°C . for $\frac{1}{2}$ hour, instead of 32.5° , which is the temperature of maximum solubility, because the transition point from the hydrated to the anhydrous form of Na_2SO_4 is at 32.75° ; and when anhydrous salt is employed at 33°C ., no hydration of the undissolved salt, and hence no error due to concentration of the solution, can occur. The difference in solubility at the two temperatures is negligible. In a water-jacketed filter, maintained at 33° , the solution is then filtered up to the mark into a 25 cc. volumetric flask without allowing it to cool, it is then washed quantitatively into a 50 cc. volumetric flask, and made up to volume. Two 10 cc. aliquots are taken for total nitrogen. The difference between this total nitrogen after allowing for the dilution, and the nitrogen of the trichloroacetic acid filtrate, is the proteose nitrogen.

25 cc. of the filtrate are pipetted into a 200 cc. Erlenmeyer flask; 25 cc. of 2.21 N sodium hydroxide and 125 cc. of 20 per cent tannic acid dissolved in 0.1 N H_2SO_4 , containing 20 per cent Na_2SO_4 , are then added in that order. The mixture is thoroughly shaken and kept at 20°C. for 4 hours. It is important to maintain the temperature near 20°C. because at temperatures only slightly higher the precipitation of peptone by tannic acid is not complete (see Table III); and at lower temperatures salt begins to crystallize out of solution, concentrating it by abstracting water of crystallization, and thereby introducing a small error. The solution is filtered, and two 50 cc. portions of this tannic acid filtrate are taken for total nitrogen. The difference between this and the previous total nitrogen gives the peptone nitrogen.

The large amount of carbon to be digested necessitates modification of the usual procedure of the Kjeldahl method. The 50 cc. of tannic acid filtrate are pipetted into a 500 cc. Kjeldahl flask and a piece of copper wire and 1 cc. of concentrated H_2SO_4 are added. The flask is then heated on the digestion rack until salt begins to crystallize out of the hot solution. It is cooled, 7 gm. of K_2SO_4 are added, and then 24 cc. of concentrated H_2SO_4 . The digestion is allowed to continue until all frothing has ceased and the solution has become freed of all suspended particles. The solution, still a dense dark brown, almost black, is cooled, 5 cc. of 10 per cent mercuric acetate are pipetted in, and the digestion is then carried to completion in the usual manner.

A portion of the tannic acid filtrate is adjusted to pH 5.0, and then pipetted into nine times its volume of 95 per cent alcohol. It is then allowed to stand for 24 hours. This hydrogen ion concentration was shown by electrometric titrations to be in the region of the isoelectric points of both proteose and peptone, including subpeptone, obtained from albumin, Witte's peptone, and Parke, Davis and Co.'s peptone. At hydrogen ion concentrations on either side of this point the precipitation of proteose, and to a greater extent of peptone, falls increasingly short of completion. After standing 24 hours the solution is filtered. To the filtrate 2 or 3 drops of saturated alcoholic solution of zinc chloride are added. After standing for a few minutes the mixture is again filtered through a dry paper. A measured amount of the second alcoholic filtrate is concentrated down on a water bath,

or, if the amounts of alcohol are too large to be lost, in Claisen flasks according to the procedure of Van Slyke. The residue is taken up again with distilled water, made up to known volume, and the total nitrogen determined.

The nitrogenous material remaining in solution, consisting probably of amino acids and the simpler peptides, has been termed the residual nitrogen.

The nitrogen in the alcohol precipitate is the subpeptone nitrogen.

II. DISCUSSION.

Trichloroacetic acid for the precipitation of protein has been employed for years; but its failure to precipitate proteoses is not so widely known. Hiller and Van Slyke (3) are of the opinion that intermediate products are precipitated by trichloroacetic acid, and Shonle and Waldo (4) have recently employed precipitability by this reagent as a criterion for proteose. In our hands, carefully purified proteose from such widely varying sources as Witte's peptone, Merck's pepsin, Eimer and Amend's pepsin, Parke, Davis and Co.'s peptone, and a peptic digest of egg albumin, failed entirely of precipitation by trichloroacetic acid. Even a derived protein, so little changed as gelatin, is not precipitated by as high a concentration of trichloroacetic acid as 30 per cent. Our results are in accordance with those of Shin Shima (5).

The conditions in which the tannic acid precipitation of peptones gives quantitative yields required careful and exact definition. The methods described in the literature, Simon (6), Henriques and Gjaldbak (7), and Plimmer (8), were found by trial to be unsatisfactory. Tannic acid was chosen because it precipitates most of the polypeptides known to be precipitable (9); and because histidine, tyrosine, and cystine, which are precipitated by otherwise suitable reagents, such as sodium tungstate, are not precipitated by tannic acid. Alcohol precipitation is undesirable because as Van Slyke and others have pointed out, alcohol precipitates are inextricably contaminated with other non-precipitable contents of the solution. Tannic acid under optimum conditions precipitated about 51 per cent of the nitrogenous material originally precipitated by alcohol.

The estimation of the amino acids was at first attempted by the usual Van Slyke amino nitrogen determination on the tannic acid filtrate before and after acid hydrolysis. Unexpectedly a reaction was found to occur between the tannic acid employed and the nitrous acid, which liberated a large amount of gas not absorbable by the alkaline permanganate. 5 cc. of tannic acid solution, containing no protein material, gave 18 cc. of gas. The nitrous acid method was clearly not feasible. Direct determination by alcohol precipitation was then resorted to. The final procedure employed was a slight modification of the methods of Folin and Denis, and Van Slyke and Meyer, for the determination of amino acids in blood.

The results for the residual nitrogen fraction by this method are probably too low, but it is the only ready method available, and for comparative work gives results, as the work of Folin and Van Slyke has shown, sufficiently indicative of the actual figures.

III. EXPERIMENTAL.

The procedure described for the complete analysis of a digest was arrived at after investigation and testing of methods for the precipitation of each of the fractions separately.

Protein Fraction.

Albumins and globulins were employed as the sources of protein. Hiller and Van Slyke found that concentrations of trichloroacetic acid above 5 per cent have some slight hydrolyzing power, and, accordingly, amounts of this reagent were used such that the final concentration did not exceed 2 per cent.

In order to test the thoroughness of this mode of precipitation, a 3 per cent solution of egg albumin (Baker and Adamson) was filtered and precipitated with trichloroacetic acid. The experiment was carried out in triplicate. 96 per cent of the protein nitrogen was found to have been precipitated, and the triplicates were in quite satisfactory agreement.

The albumin on subsequent analysis was found to contain 94.5 per cent protein, 4.4 per cent proteose, and 1.1 per cent peptone.

In order to determine whether temperature variations over a normal range have any effect on the degree of precipitation, a 5 per cent solution of egg albumin (Merck) was precipitated in 2 per cent trichloroacetic acid at 10°, 15°, 20°, and 25° ($\pm 0.5^\circ$). Again, approximately 96 per cent was

6 Fractional Analysis of Protein Digests

precipitated and the results showed that temperature changes, within the above range, are without effect on the degree of precipitation of protein by trichloroacetic acid.

Metaprotein Fraction.

The following experiments indicate that metaprotein is also precipitated quantitatively by trichloroacetic acid.

A solution of Lieberkühns jelly was made in dilute HCl and filtered, giving a clear filtrate, containing 10.4 mg. of metaprotein N in 10 cc. This filtrate was neutralized to litmus, producing a filterable suspension, which was divided into two portions, *A* and *B*. *A* was filtered and to the filtrate sufficient 20 per cent trichloroacetic acid was added to give a final concentration of 2 per cent. It was allowed to stand for 5 minutes, filtered, and the total nitrogen of the filtrate determined. To *B*, with no previous filtration, trichloroacetic acid was added directly and it was then allowed to stand for 5 minutes, after which it was filtered and the total nitrogen of the filtrate determined. In *A*, metaprotein was removed by filtration, after neutralization, before the addition of the trichloroacetic acid, leaving a considerable residue of protein, as shown by the subsequent precipitate with trichloroacetic acid. On comparing the total nitrogen of the final filtrates from *A* and *B* they were found to be identical.

Proteose Fraction

The quantitative precipitation of proteose involves inconveniences not encountered in the trichloroacetic acid precipitation of protein. Ammonium sulfate cannot be used on account of its nitrogen content; and Na_2SO_4 , which is employed instead, for quantitative precipitation, involves filtration at 32.75°C . (10). It is desirable also to use a specimen of salt with as low and as constant a water of crystallization as possible. The commercial c.p. anhydrous salt is quite suitable, and the volume changes due to the addition of the Na_2SO_4 can be determined in controls and the necessary corrections estimated.

To test the possibility of quantitative precipitation of proteose in this way, 5 cc. of 5 per cent proteose, obtained from Parke, Davis and Cosl. peptone by salting-out with Na_2SO_4 , were pipetted into each of three test-tubes, to which were added 3 gm. of Na_2SO_4 , and kept at $32-34^\circ$ for 1 hour, with frequent stirring. The solutions were then filtered directly into 500 cc. Kjeldahl flasks and the precipitates washed three times in the funnels with saturated Na_2SO_4 at 32.5°C . Total nitrogen estimations were carried out in the usual way on these filtrates and on the original solution.

The precipitation was practically complete, 99.2 per cent of the proteose was precipitated, and the checks were in good agreement.

In the actual working routine the procedure is somewhat different. The solution saturated with Na_2SO_4 at 33°C . is filtered at the same temperature, using a filtration box (see below), into a 25 cc. volumetric flask also maintained at 33°C . When 25 cc. of filtrate have been obtained the flask is removed and its contents are immediately washed quantitatively into a 50 cc. volumetric flask and made up to volume. At this dilution the solution is just under saturation at room temperature (20°) and can be pipetted without clogging the point of the pipette. With this procedure, after allowing for the dilution, 99.1 per cent of the proteose was removed.

For the convenient carrying out of this filtration a piece of apparatus, the filtration box mentioned above, was constructed. This consists essentially of two copper boxes, one above the other, connected by two side walls and a back of sheet copper. The upper box is a battery of four hot water

TABLE I.

Effect of Varying C_{H^+} on the Precipitation of Proteose by Sodium Sulfate.

pH	Total N.	N after filtration.	Removal.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
3.0	87.2	2.0	97.7
4.0	87.2	2.0	97.7
5.0	87.2	2.0	97.7
6.0	87.2	2.0	97.7
6.6	87.2	2.0	97.7
9.2	79.3	1.8	97.7

funnels of suitable dimensions, maintained at 33°C . by a micro burner, with a steady stream of air bubbling through the water for mixing purposes. The flasks into which the liquid is filtered, stand in the lower box, in water, also maintained at 33°C . The depth of this box is such that the bulb of the 25 cc. volumetric flask may be kept under water. The flasks are held in position by a lid of sheet copper, which has four narrow slots, each just wide enough to admit the neck of a flask. The stems of the funnels project for about 1.5 inches into the necks of the flasks.

It was necessary to ascertain the influence, if any, of the hydrogen ion concentration on the degree of completeness of precipitation of proteose by Na_2SO_4 . Proteose from Parke, Davis and Co.'s peptone, thrice precipitated and washed, was dissolved in distilled water and brought to a number of different hydrogen ion concentrations, maintaining the same dilution in all. The precipitation was carried out as described above. The results are given in Table I.

The degree of precipitation by Na_2SO_4 is clearly independent of the hydrogen ion concentration. This result was expected because it is unlikely that "salting-out" is either an isoelectric point phenomenon, or a precipitation as an insoluble salt. The consistency of the nitrogen content of the filtrate indicates probably an impurity. However, even if it represents a soluble residue, the smallness of the amount, and its constancy, testify to the serviceability of the method, with proper controls, for quantitative work.

The allowance for the dilution, and for the increase of volume due to heating to 33°C ., is made by noting the increase in volume of water at 20°C ., saturated with Na_2SO_4 at 33°C .. This is carried out in a 50 cc. volumetric flask, with a long graduated neck, containing 50 cc. to the base of the neck.

The following is a typical example of the order of magnitude of the increase. 50 cc. of water at 20°C ., saturated with Na_2SO_4 at 33°C ., occupied 53.5 cc. Since only 25 cc. are employed in the analysis, and are diluted to 50 cc., the value of 1 cc. of diluted filtrate is $\frac{50.0}{53.5} \times \frac{1}{2} = 0.467$ cc.

Peptone Fraction.

For the determination of the optimum conditions for peptone estimation by tannic acid, after preliminary experiments, the various factors which might affect the degree of precipitation were separately investigated. After each precipitation, total nitrogen estimation was carried out on the filtrate. The first factor investigated was the influence of the hydrogen ion concentration. The varying conditions and their results are summarized in Table II.

The pH in every case was estimated electrometrically in duplicate. The mixtures were allowed to stand for 24 hours at room temperature before filtration. The results show that there is a definite optimum pH in the neighborhood of pH 7.0.

The experiment was repeated with twice the final concentration of tannic acid and optimum precipitation was again found to occur at exactly the same reaction, *viz.* pH 7.0, the results being similar to those shown in Table II.

Incidentally a curious phenomenon was observed, which seems worth noting; *viz.*, that on exposure to air, the tannic acid solutions became darker and curiously more acid, not unlike the behavior of hemoglobin on oxidation.

It is desirable, for several reasons, to use as little tannic acid as possible in this operation and accordingly the minimum concentration of tannic acid necessary for maximum precipitation was next explored. The minimum concentration was found to be about 14 per cent which effects a removal of 44 per cent of the nitrogen of Parke, Davis and Co.'s peptone remaining in

TABLE II.
Effect of C_H^+ on Precipitation of Peptone by Tannic Acid.

Peptone solution.	20 per cent tannic acid in 0.1 N H_2SO_4 .	0.221 N NaOH.	H_2O	pH	N in filtrate.
cc.	cc.	cc.	cc.		mg.
5					31.2
5	25	0	70	2.7	30.8
5	25	10	60	3.7	30.8
5	25	20	50	4.7	29.4
5	25	30	40	5.9	27.9
5	25	40	30	6.6	27.1
5	25	50	20	7.1	26.7
5	25	66	14	7.6	29.4
5	25	70	0	7.6	29.6

solution after precipitation of the proteoses by Na_2SO_4 . A precipitation carried out simultaneously according to the procedure of Henriques and Gjaldbak (7) removed only 20 per cent.

The experiments were all carried out in the summer at room temperature. One hot evening it was found, that under conditions so far considered optimal, very little precipitate was obtained. Cooling under the tap caused immediate precipitation. Evidently temperature is an important factor that cannot be disregarded.

Five portions of the peptone solution were precipitated with the optimum concentrations of tannic acid and alkali at 10°, 15°, 20°, 25°, and 29° ($\pm 0.5^\circ$). The solutions were maintained at these temperatures for 3 hours and then filtered.

The N in the filtrate was determined with the results recorded in Table III.

It is obvious from these results, that the tannic acid precipitation should not be carried out at temperatures higher than 20°C. It was found that 3 hours standing was only very slightly, if any, less effective than 24 hours. With 24 hours standing the nitrogen content of the filtrate from a precipitation carried out in the same manner as the 20°C. experiment above, was 34.7 mg.; with 3 hours standing, 35.2 mg.

To test if precipitation had been complete, solutions of peptone of various concentrations were precipitated with tannic acid solutions under the optimum conditions so far worked out, with

TABLE III.

Effect of Temperature on Precipitation of Peptone by Tannic Acid.

Temperature.	Total N in filtrate.	Removal.
°C.	mg.	per cent
Control.	79.2	
10	32.9	58.4
15	32.7	58.4
20	33.0	58.3
25	28.4	51.5
29	79.2	0.0

the expectation that the same percentage of the total nitrogen would be precipitated out of each solution. The most concentrated solution of peptone was saturated with Na_2SO_4 at room temperature, and the other solutions were made up from it by dilution with water. The results are given in Table IV.

The difference in the percentage removal between the last two samples with a salt concentration of 145 gm. per liter was probably due to the dehydration caused by the crystallization of salt which occurred in the second of these and which, therefore, would increase the concentration of the dissolved substances there. The results shown in Table IV compelled investigation of the effect of salt concentration.

Peptone was prepared from Parke, Davis and Co.'s peptone, in the usual manner, by salting-out with Na_2SO_4 at 33°C. The concentrations of salt were varied by adding varying amounts of Na_2SO_4 to the 0.1 N H_2SO_4

in which 20 per cent of tannic acid was dissolved. Optimum precipitation was found to occur when the tannic acid solution employed contained 20 gm. of Na_2SO_4 in 100 cc. The degree of precipitation was then independent of the peptone concentration.

After this experiment it was thought that possibly a lower concentration of tannic acid might be employed, especially as a great deal of sodium tannate in addition to peptone tannate is precipitated when 14 per cent is employed.

With peptone solution from Parke, Davis and Co.'s peptone, and 20 per cent tannic acid dissolved in 0.1 N H_2SO_4 containing 20 per cent Na_2SO_4 ,

TABLE IV.

Effect of Salt Concentration on Degree of Precipitation of Peptone by Tannic Acid.

Salt concentration.	Total N.	N in filtrate.	Removal.
<i>gm. per l.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
120	94.5	41.6	56.0
60	72.1	32.7	54.6
30	47.0	24.9	46.9
15	24.7	15.3	38.1
145	94.5	35.6	62.3
145	24.7	9.7	60.9

TABLE V.

Fractional Analysis of a Known Mixture of Products of Peptic Hydrolysis.

	Calculated per cent of total N.	Experimental.			
		Per cent of total N.			
		1	2	3	4
Protein.....	9.7	9.9	9.7		
Proteose.....	18.3	18.3	18.3		
Peptone.....	40.4	39.4	40.4	39.8	39.6
Subpeptone.....	31.6	32.4	31.6	31.3	31.6

mixtures were made up with the final tannic acid concentration varying from 7 to 14 per cent, and the precipitation was carried through under optimal conditions.

The results showed that on the whole the conditions for optimum precipitation were those arrived at previously.

With the completion of this experiment, all the possible factors had been taken into account: reaction, concentration of tannic acid, temperature, time, concentration of salt, and concentration of peptone.

A mixture was then made containing measured amounts of protein, proteose, peptone, and subpeptone, and analyzed by the method described on page 1, which is based upon the methods worked out for each of the fractions separately. The results are given in Table V.

Nos. 1, 2, and 3 were carried out on a total nitrogen content of 237.6 mg.; No. 4, on 207.9 mg.

TABLE VI.

Fractional Analysis of Peptic Hydrolysates in Presence of Varying Amounts of Pepsin of Known Fractional Composition.

	Pepsin.	Total N of digest after pepsin N was subtracted.	Per cent of total nitrogen.			
			Protein.	Proteose.	Peptone.	Sub-peptone.
	<i>gm.</i>	<i>mg.</i>				
A		269.1	8.4	31.7	34.7	25.3
B	0.51	294.5	8.4	31.8	35.9	23.7
A		345.5		38.1	32.1	30.3
B	1.03	345.5		38.1	32.4	30.3
A		470.0	1.3	32.0	35.6	31.0
B	1.1	536.0	1.0	32.0	35.9	31.3
A		307.1		38.1	32.1	29.8
B	1.0	337.8		38.3	30.5	31.4

TABLE VII.

Relation of C_H^+ to Amount of Material Precipitated in 95 Per Cent Alcohol.

Proteose.		Peptone.	
pH	Weight precipitated from 10 cc. solution.	pH	Weight precipitated from 10 cc. solution.
	<i>mg.</i>		<i>mg.</i>
2.1	139	1.5	6
2.4	144	1.7	16
2.9	156	1.9	53
3.4	159	2.5	81
4.3	167	3.4	102
5.3	167	4.2	129
7.6	159	5.0	140
8.3	156	6.9	122
9.5	155	8.4	99

More complete confirmation of the accuracy of the method was provided by the following series of analyses. Eimer and Amend pepsin was analyzed, and it was found that 2.00 gm. contained 259.9 mg. of nitrogen; that it was free of protein nitrogen, and contained 31.0 per cent as proteose, 42.2 per cent as peptone, and 26.8 per cent as subpeptone. A number of samples from several prolonged and concentrated peptic digests were ana-

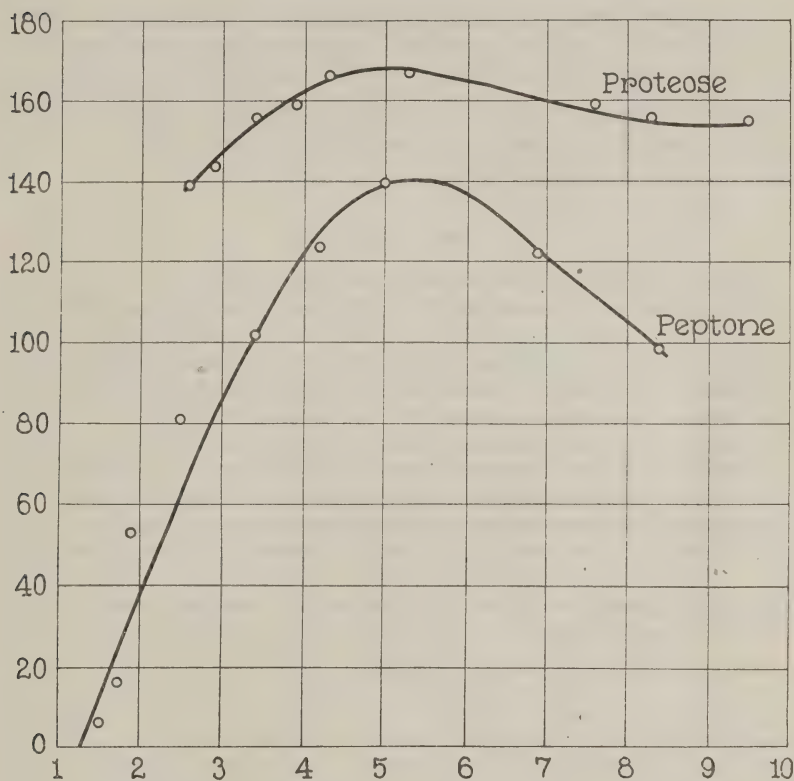


FIG. 1. Relation between pH and amount of proteose and peptone precipitated from 10 cc. of 2 per cent solution in 95 per cent alcohol. pH given as abscissæ; ordinates, amount precipitated in mg. of dry weight.

lyzed, half of which, A, contained no additional pepsin, and the other half, B, varying amounts. The values of pepsin corresponding to the above were subtracted from the analytical results obtained with B, and the final data compared with the results of A. The correspondence, as Table VI shows, is quite good. The widest varying pair, out of eighteen such analyses, carried out over a period of 5 months, is the last two given. The largest

error is in the peptone and subpeptone fractions where the titration result is multiplied by approximately 16, while the results for protein and proteose are multiplied only by 8; and all four are expressed in terms of percentage of the same original total nitrogen.

Residual Nitrogen.

The dependence of the degree of precipitation of proteose and of peptone by alcohol upon the hydrogen ion concentration is shown in Table VII and in Fig. 1. The phenomenon is similar to the precipitation of protein in alcoholic solution by small amounts of salt, which, as Loeb (11) has pointed out, is strongly indicative of the existence of a Donnan equilibrium.

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THE ENZYMATIC SYNTHESIS OF PROTEIN. I.

THE SYNTHESIZING ACTION OF PEPSIN.

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I. HISTORICAL.

A condition in which a synthesis of protein-like material occurs, was first arranged in 1886 by Danilewski (1) who observed the formation of a precipitate when stomach extract was added to a concentrated solution of the products of peptic hydrolysis. He considered the causative agent to be an enzyme, because precipitation did not occur if the stomach extract had been previously heated to 100°C. This result was confirmed in 1895 by Okunew. Both Danilewski and Okunew concluded that the reaction involved synthesis of the products of protein decomposition into a more composite molecule approaching in complexity a native protein.

In 1901 Sawjalow (2, 3) investigated the phenomenon more extensively and named it plastein formation. He recognized the necessity for a suitable degree of acidity though he did not define it precisely.

Using Witte's peptone in a concentration of 27.2 per cent, Sawjalow obtained 13.6 per cent of the total dry weight in the precipitate. He attempted an estimation of the molecular weight of plastein by means of acid and alkali titration and arrived at an average value of 6,087.

Sawjalow and other workers, Lawrow and Salaskin (4), Kurajeff (5), and Bayer (6) sought to discover the source of plastein among the various fractions obtained by the method of Pick from a protein digest, but failed to reach any agreement.

Bayer reached the conclusion that proteoses took no part in the synthesis of plastein.

A. E. Taylor (7, 8) and T. B. Robertson (9, 10) have reported synthesis of protein by means of trypsin and pepsin.

In Taylor's experiments the synthesis was accomplished after 5 months standing and the yield was very small. In Robertson's experiments the yield was larger and more rapidly obtained. Robertson found indications which led him to assume the existence in pepsin of separate hydrolyzing and synthesizing components. Our experiments indicate that this assumption was possibly erroneous.

Bayliss (11) in a criticism of the results of Robertson considers that he was dealing with a colloidal precipitation phenomenon and not with an enzymatic synthesis.

The last contribution to the problem of the enzymatic synthesis of protein appeared in 1911 and 1912 (12, 13) by Henriques and Gjaldbak. They obtained plasteins by means of trypsin and pepsin from partial acid and alkali hydrolysates and with trypsin from peptic digests. They made the important observation that neither enzyme was capable of producing plastein from any type of digest of gelatin; nor from any tryptic digest except of casein, and here only by pepsin. There is indicated in their work the possibility of the influence of dilution, of temperature, and of acidity on the plastein formation. In their final experiments they point out an anomalous simultaneous hydrolysis and synthesis. Their important contribution was the estimation of the ratio of the free amino to the total nitrogen in plastein, which they found to be lower for plastein than for any of its precursors. These results afforded the only evidence for assuming the complexity of the plastein molecule to be greater than that of the substances from which it is built; their additional observations of a decrease in formol titratable nitrogen, simultaneous with an increase in the tannic acid precipitable nitrogen, are not as significant, because the titration results do not parallel those from the tannic acid precipitation, and because they employed concentrations of tannic acid too dilute to give complete precipitation. The problem of the identity of the enzyme responsible for plastein formation was not attacked; nor are their results capable of any general kinetic interpretation, because it is probable from the high ratios of free amino nitrogen to total nitrogen in their peptic digests, 38 per cent, and the high ammonia liberation, 5 per cent, that their pepsin preparation contained in addition to pepsin, other enzymes of ereptic and autolytic types.

Summing up, the only unchallenged synthesis of protein is that of protamine by trypsin, attained by Taylor. The extreme slowness of this reaction and the paucity of the yield, despite the simplicity of the protein hydrolyzed, inspired little confidence of any probable physiological significance. The work on plastein, as a study of enzymatic synthesis, appears to have been virtually dismissed as an unphysiological and anomalous phenomenon. There have been no further contributions since the work of Henriques and Gjaldbak in 1912. The mechanism by which the synthesis of protein is effected in the tissues is still only conjectural, despite the strong implications from mass law and thermodynamic reasoning that the conditions of plastein formation are the very conditions under which the synthesis occurs in the tissues.

II. EXPERIMENTAL.

In a concentrated solution, approximately 40 per cent, of the products of peptic hydrolysis of egg albumin (Merck, U. S. P.), whose reaction had been adjusted to pH 4.00,¹ pepsin brought about a precipitation of material which, on investigation, proved to be of the order of complexity of native protein.

Proof that this precipitate, which for the sake of historical continuity has been named plastein, is more complex than the substratum from which it is produced, is afforded by direct evidence from a number of sources.

First, the ratio of free amino to total nitrogen is lower in plastein than in the most complex product of the digest from which the plastein was synthesized. This was first observed by Henriques and Gjaldbæk, and has been here confirmed.

A suspension of plastein in distilled water was boiled for half an hour to destroy enzymes possibly carried out of the digest from which it was produced. It was then washed three times by decantation and three times on a filter with a stream of distilled water. A suspension of pure plastein was thus obtained, free of any adherent matter, which was very fine in texture, and grayish white in color. A suspension was made in 0.07 N HCl, and the amino nitrogen in 5 cc. determined in duplicate in the macro Van Slyke apparatus. The plastein was quite soluble in the nitrous acid; and the 5 cc. of suspension gave 1.12 mg. of free amino nitrogen. The total nitrogen of 5 cc., estimated by the macro Kjeldahl method, was 16.9 mg. The ratio, therefore, is

$$\frac{\text{Free amino N}}{\text{Total N}} = \frac{1.12}{16.9} = 6.6 \text{ per cent}$$

Proteose, obtained by salting-out with Na₂SO₄ from the same digest from which the plastein was synthesized, was twice reprecipitated and then redissolved. Assayed for free amino nitrogen, 5 cc. gave 4.39 mg. The total nitrogen of 5 cc. was 42.0 mg., therefore

$$\frac{\text{Free amino N}}{\text{Total N}} = \frac{4.39}{42.0} = 10.5 \text{ per cent}$$

¹The experimental justification for this procedure will be given in a future paper.

Since the increase of the free amino nitrogen of a protein digest has often been taken as a measure of the degree and rate of hydrolysis, a decrease in the ratio of free amino to total nitrogen may justifiably be interpreted as proof of synthesis. With this criterion, therefore, plastein proves to be a synthetic product. The figures of Henriques and Gjaldbæk, obtained by the formol titration, indicate that the complexity of the plastein here obtained, is of approximately the same order as of many native proteins. These figures for a number of proteins, together with some determined by the authors, are given in Table I.

This criterion, however, cannot be trusted too far. The figures for casein would lead to the improbable conclusion that this protein is of lower complexity than a product of digestion of egg albumin; *viz.*, proteose. However, in the case of two substances

TABLE I.

Substance.	Free amino N.
	Total N
Edestin.....	2.8
Egg albumin.....	6.7
Casein.....	11.9
Fibrinogen.....	7.1
Serum globulin.....	6.0
Witte's peptone.....	14.4
Plastein*.....	6.6
Egg albumin*.....	3.5
Proteose*.....	10.5
Peptic digest of plastein*.....	17.5

* These values obtained by the authors with the Van Slyke method.

isolated from the same digest, differences in the value of this ratio must indicate corresponding differences in complexity.

Proof of an entirely different nature of the occurrence of synthesis here, is afforded by the results obtained from the fractional analysis of originally identical solutions, in which varying amounts of plastein had been formed. The analytical procedure has been given in detail in the previous paper. Regarding solely the results given in Table II, it is evident at once that all fractions of the peptic digest have contributed to the formation of plastein; and in approximately the same relative amounts as they are obtained in the peptic hydrolysis of albumin. These results show the fallacy of the search of Salaskin, Kurajeff, Bayer, Lawrow, and

others for the source of plastein in any one component of a digest, and afford striking proof of the synthetic nature of plastein.

The highest amount of plastein noted above, 27.3 per cent, falls considerably short of the amounts obtained later, when the optimum conditions in regard to temperature and reaction, enzyme and digest concentrations, had been located. Then, yields as high as 39 per cent were found repeatedly. A fuller description of the investigations by which these conditions were arrived at, will be published later.

TABLE II.

Plastein N in per cent of total N.	Proteose N in per cent of total N.	Peptone N in per cent of total N.	Subpeptone N in per cent of total N.
Series I.			
1.3	32.0	35.6	31.0
3.2	32.8	33.2	31.0
7.1	30.5	32.8	29.8
17.2	24.2	29.2	29.4
18.7	23.0	29.4	28.7
27.3	19.6	28.1	25.0
Series II.			
1.0	32.0	35.9	31.3
1.4	32.2	34.9	31.6
6.9	30.7	33.1	29.7
16.0	27.0	28.4	28.7
23.3	21.8	27.2	27.8
Series III.			
3.2	28.2	37.1	31.6
25.3	17.7	29.9	27.1

Proof in still another direction of the protein nature of plastein is obtained from the characteristic protein color of the biuret test given by this substance. Proteose (the most complex constituent of the digest), the whole digest, and a peptic hydrolysate of plastein itself all gave a color of distinctly different quality, the characteristic pink or rose shade. The color of the biuret gives a fair indication of the complexity of the substance causing the color. This pink nuance of the proteose is easily distinguishable from the mauve of protein. In addition, plastein

is precipitated from its solution in either concentrated or dilute solutions of strong acid, by trichloroacetic acid. In view of the absence of any effect of this reagent upon gelatin, or upon any of the proteoses, taken in conjunction with the other evidence quoted above, there can be little room for doubt that plastein is a product of a synthetic reaction, and that its complexity is of the order of a native protein. The similarity to native protein is further confirmed by the readiness with which it is digested by pepsin, at a similar hydrogen ion concentration, giving the same products, and with the same velocity.

A fairly heavy suspension of plastein was adjusted with 0.07 *N* HCl to a reaction where $\text{pH} = 1.7$. To a total volume of 250 cc., 0.5 gm. pepsin (Merck) was added, and the suspension was set away in the incubator at 37°C. In 1 hour all of the plastein had gone into solution. 22 hours later the pH had risen to 2.2, and on saturation with Na_2SO_4 at 33°C. a voluminous crop of proteose floated to the surface.

In another experiment, a freshly prepared plastein was washed on a filter, suspended in water, then boiled for half an hour; cooled, decanted three times with distilled water, and then washed three times on a filter with distilled water. A suspension was made in HCl, giving a final reaction where $\text{pH} = 1.7$. The free amino nitrogen of 5 cc. was found to be 1.29 mg. To 50 cc. of this suspension (*A*) 0.05 gm. of pepsin (Merck) was added.

To another 50 cc. (*B*) 0.05 gm. of boiled pepsin was added. Both were set away at 37°C. In 2½ hours *A* was clear and *B* was unchanged. 26 hours later the free amino nitrogen in 5 cc. of *A* was 2.8 mg.; and of *B* 1.37 mg. The slight rise in *B* was probably due to the commercial pepsin added, which is known to consist chiefly of the products of digestion of protein, and to the slight hydrolyzing effect of the acid. The ratio of free amino N to the total N in *A* was 17.1 per cent.

The ratio for proteose is 10.5 per cent; therefore, besides proteose, large amounts of even simpler products were produced on the hydrolysis of plastein, as is the case with protein.

No hydrolysis was obtained when proteose, salted-out from a digest from which plastein had been synthesized, was submitted to the action of pepsin.

Proteose from a peptic digest of egg albumin, which gave no precipitate with saturated copper acetate, was redissolved in water, boiled, filtered, and reprecipitated with Na_2SO_4 . It was then redissolved in HCl, adjusted to pH 1.7, and 0.12 gm. of pepsin (Merck) was added. The free amino nitrogen of 5 cc. was 4.40 mg. 24 hours later this value for amino nitrogen

was unchanged. The total nitrogen was 42.0 mg., giving a value for the ratio of free amino to total nitrogen of 10.5 per cent.

The conclusion is, therefore, that it is possible for pepsin to synthesize a linkage, or linkages, which under other conditions it hydrolyzes.

Excessive quantities of enzyme are unnecessary for the synthesis of plastein. Pepsin in 8 per cent concentration gave rise to 33.2 per cent plastein; while in the same solution of products, the concentration of enzyme preparation usually employed in hydrolysis, *viz.* 0.1 per cent or 80 times less, gave 13.1 per cent plastein in the same length of time.

The properties of the plastein prepared resemble those of a protein denatured by dilute acid. It is only very faintly soluble in neutral water, but is quite soluble in dilute strong acids and alkalis, more so in the latter than in the former. A region of distinct solubility exists between pH 8.8 and 9.9, with a maximum solubility at pH 9.2. At hydrogen ion concentrations somewhat greater than pH 1.0, it is fairly soluble, but is quite insoluble at all pH values between 2.0 and 7.0. It is, therefore, insoluble in 0.07 N acetic acid. It is only slightly soluble in 10 per cent NaCl, but very soluble in strong HNO₃ and strong HCl. It is not soluble to any noticeable extent in 95 per cent alcohol.

It is incompletely precipitated from its solution in 0.025 N NaOH by the addition of NaCl to half saturation at room temperature; a much heavier precipitate is obtained on saturation. Boiling coagulates the plastein in alkaline, but not in acid solution. It is precipitated from its solution in either strong or weak HCl by trichloroacetic acid. It gives, as mentioned above, a characteristic protein biuret, quite distinct from a proteose biuret, and gives a characteristic, positive, Heller's test. If allowed to stand in concentrated HCl a purplish color develops.

It is rapidly digested by pepsin at pH 1.7.

A possible cause of the insolubility of plastein in neutral solutions is indicated by a phenomenon, which was encountered incidentally, and which demonstrates the inability of ordinarily soluble albumin to exist in a soluble form, under the conditions in which peptic synthesis occurs. To 4 cc. of a concentrated peptic digest of albumin at pH 4.0, 2 cc. of 5 per cent albumin solution

were added. A precipitate formed immediately, which increased on standing. After 1 hour at 38°C. the mixture was filtered; and on testing the filtrate with trichloroacetic acid, no precipitate was obtained, indicating the absence of any protein in solution. The total nitrogen of the precipitate was equal to the nitrogen of the protein added. The precipitate, after thorough washing, remained quite insoluble in water, and exhibited the same solubility properties as plastein. The digest contained no active enzymes, because it had previously been boiled for an hour. It seems that the soluble protein on coming into contact with the solution of products at pH 4.0 was immediately denatured, or at any rate was rendered insoluble.

The phenomenon is under thorough investigation.

The preliminary results of the first cursory examination are given below. At least two factors are of significance, the hydrogen ion concentration, and the concentration of products.

With acidities up to pH 5.4 the precipitation of protein went to completion. At hydrogen ion concentrations lower than this, a turbidity only was obtained, which did not increase on prolonged standing (1 week) at 38°C.

It was found that in solutions of products under 5 per cent protein is not precipitated. On the other hand albumin remains in solution in concentrations of products as high as 30 per cent, when the hydrogen ion concentration is at pH 7.0.

An experiment was carried out to ascertain if, with the precipitation of the albumin, the cause of the precipitation was removed. A mixture of albumin and digest was filtered after the first precipitation, and to the filtrate a second portion of albumin was added. Addition of trichloroacetic acid had shown that the first precipitation was complete. The precipitate formed again with the second portion of albumin, and no less quickly. This procedure was repeated twice more, and in both cases there again occurred the same speedy formation of the precipitate. After this fourth addition of albumin, the concentration of products, which originally had been 9 per cent, had now been reduced to 4 per cent. The experiment was, therefore, not carried any further because at 4 per cent the denaturation and precipitation of albumin no longer takes place. The results obtained, however, showed that in this precipitation of albumin there is no appreciable, if any, removal of the material which causes it.

In view of the well known exceptional behavior of gelatin in nutrition, an attempt was made to effect a synthesis of protein in

a concentrated peptic digest of gelatin. Henriques and Gjaldbæk have published the failure of this reaction in their experiments with every type of gelatin digest. But with our more definite knowledge regarding the optimal conditions for peptic synthesis, on the one hand, and the nutritional deficiencies of gelatin, on the other, it was thought that, despite their previous failure, a probability of success existed now, which warranted another attempt.

Accordingly a 6 per cent solution of gelatin was digested at pH 1.6, at 37°C. for 3 weeks. It was then filtered, heated at 80–90°C. for 1 hour to destroy the enzyme, and then concentrated *in vacuo* to a concentration of approximately 50 per cent. The resulting solution was clear, reddish brown, and somewhat viscous, but was pipetted without difficulty. It was adjusted to pH 4.0. To 10 cc. 0.5 gm. of Merck's pepsin was added, rubbed into solution, and the mixture set away with chloroform, tightly stoppered, at 37°C. At the end of a week the solution was still quite clear and did not give a precipitate either with trichloroacetic acid or on neutralization. Evidently synthesis of plastein, or of any other protein body had failed.

TABLE III.

No.	Gelatin digest. pH 4.0	Albumin digest. pH 4.0	Tyrosine.	Trypto- phane.	Pepsin.	Plastein N.
	cc.	cc.	gm.	gm.	gm.	mg.
1	10		0.09		0.5	0
2	10		0.09	0.09	0.5	0
3	10			0.09	0.5	0
4	5	5			0.5	92
5	0	10			0.5	230

This, however, from the nutritional behavior of gelatin might have been predicted. Quite another result could be expected in the presence of tryptophane. With this in view five flasks were prepared. The materials were added to each, and the results are given in Table III. The amino acids were first dissolved in 0.07 N HCl, to which the enzyme was first added, and then the gelatin digest. All the mixtures were set away with chloroform, tightly stoppered, at 37°C. for 3 days. During this period tyrosine crystallized out of solution.

The analysis for plastein was carried out in the usual manner.

As the figures in Table III show, synthesis failed also in the presence of the amino acids. The results in Nos. 4 and 5 are of

special interest. The experiment was carried out to test if in the synthesis of the products of the albumin digest, some of the hitherto unsynthesizable gelatin radicles could be carried into the plastein molecule. If this had occurred the plastein in No. 4 would have been more than half the amount in No. 5, because the synthesizable albumin material in the former was exactly half the amount of the latter. Less than half the plastein in No. 5 was found in No. 4.

The gelatin radicles were thus found to be incapable of taking part in the synthesis. Not only this, they exerted apparently a slight inhibitory influence on the synthesis of the albumin fractions.

Quite another conclusion is possible if it be assumed that the occurrence and extent of synthesis is dependent upon the existence in the digest of one essential substance, without which the reaction cannot occur. When this substance is completely utilized by its inclusion in the plastein molecule synthesis comes to a stop. In gelatin digests this substance is absent; and, moreover, it is neither tyrosine nor tryptophane. Beyond this superficial speculation it does not appear advisable to proceed; though most of the quantitative aspects of plastein synthesis are not incompatible with it. It was thought worth while only to point out this quite different possible explanation, which if true is of significance.

III. THEORETICAL.

In applying the mass law to the case of hydrolysis of protein the equation is written



and from this equation it has generally been assumed that sufficient diminution of the concentration of water, in the absence of any great concentration of protein, would cause the reaction to proceed from right to left. The fact is usually overlooked that the molecular concentration of the water is enormous when compared with the concentration of the other substances and, therefore, even with tenfold concentration of the solution the ratio of the molecular concentrations is so little altered that reversion, by this mechanism alone, would be practically impossible *in vitro*.

The opinion is occasionally met with, that protein synthesis must be especially difficult, for reasons based on the fact that the hydrolysis of proteins goes to practical completion. The equilibrium point is apparently at 100 per cent decomposition, the catalyst is assumed not to influence the point of equilibrium and reversion of the reaction is consequently difficult of accomplishment.

To account for the fact of synthesis in the tissues, there have been postulated the influence of surfaces for the production of a concentration of water so low that molecularly it would be of the same order of magnitude as the concentration of the products and the protein. An example of this reasoning is found in Bayliss' "Principles of general physiology" (14). Beginning with an alcohol-water-acid-ester system, the usual $\frac{K_1}{K_2} = K$, the equilibrium constant, is deduced; where K_1 (ester) (water) = K_2 (alcohol) (acid). He then states:

"Put in this form, we see that if we increase one component, the result must be to decrease its fellow, since the value of the fraction *must remain unaltered*. Suppose we increase water, the value of this fraction can only be kept constant either by increasing (alcohol) (acid) or by decreasing (ester). In point of fact, of course, the two are identical, since one cannot take place without the other. The results of excess of water should be, therefore, to increase the hydrolytic reaction of the system, as found by experiment.

"The conclusion to be drawn from this fact is that, in order to obtain much indication of the synthetic aspect of enzyme action, the concentration of water must be diminished as far as possible.

"In the living cells, where synthetic processes readily take place, it seems that there must be some very effective means of doing this, perhaps by surface condensation or inhibition on the part of the colloids. But as yet we have no clear idea of the mechanism."

In the above example the molecular concentration of water is of the same order of magnitude as those of the other reacting components. In the protein systems usually encountered *in vitro*, however, and also in the tissues, if no special mechanisms are postulated, the molecular concentration of water must be enormously greater than that of the reacting components. If the essential conditions for synthesis were those demanded by the Bayliss example, reversion would, *in vitro*, be impossible of attainment,

because the enormous value of the molecular concentration of water would always drive the reaction from left to right.

To consider the 100 per cent decomposition of protein as "equilibrium" is incorrect, because one of the components on the left-hand side of the equation, *i.e.* water, is at relatively infinite concentration. In order to account for the occurrence of synthesis in the tissues it would, then, be obligatory to postulate, as Bayliss does, a special mechanism capable of effecting enormous diminution of the concentration of water, with the resulting enormous increase in concentration of the substances on the right-hand side of the equation.

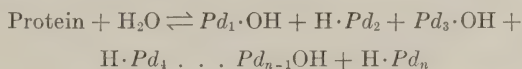
But in the hydrolysis of protein, we have the formation of a number of components from 1 molecule of protein. This fact, overlooked in Bayliss' discussion, places the hydrolysis and synthesis of protein, and to a lesser degree of fats, in entirely another category.

Moore (15) by thermodynamic reasoning arrived at a conclusion, which can be paraphrased as follows: There is a constant relationship at equilibrium between the osmotic pressure of the substance undergoing decomposition, and the n th power of the osmotic pressure of any one of the products split off, where n is the number of molecules arising from 1 molecule of undecomposed substrate. The equation is $P_a = K.P_b^n$. The greater the value of n , the greater is the tendency for synthesis in concentrated solutions and for hydrolysis in dilute solutions. In confirmation of this general theorem Moore quoted experiments of Croft Hill, who has shown that the ferment of malt in concentrated solutions of glucose forms a disaccharide, and that if the solution contains less than 4 per cent of glucose the synthesis of the disaccharide does not occur. In quite dilute solutions of maltose, its hydrolysis is known to proceed to completion.

Since the heat of reaction of hydrolysis of proteins is very low, and the value of n is high, from thermodynamical reasoning reversibility is predictably possible in two ways, by increasing the concentration of the substances in solution (for our purposes the products of hydrolysis) or by raising the temperature. Thermodynamic possibility, however, except in an extremely rigid sense, does not insure the occurrence of a reaction. If, for instance, a dilute solution of protein is maintained at a reaction where pH

=6.0, pepsin is unable to effect any hydrolysis (16); nor does the protein in the absence of pepsin suffer a considerable degree of hydrolysis, at low temperatures, merely as a result of acidity of this strength. At pH 1.7, however, in the presence of pepsin, the hydrolysis proceeds rapidly. Exactly the same necessity for suitable conditions, over and above the thermodynamic possibility, prevails in the case of reverse reaction; *i.e.*, synthesis.

The equation for the hydrolysis of protein may be written:



where n is the number of molecules into which 1 molecule of protein is split, and Pd is the symbol for one of these products. The equilibrium equation becomes, if the molecular concentration of water may be considered constant,

$$\frac{(\text{Pd}_1 \cdot \text{OH} \times \text{H} \cdot \text{Pd}_2 \dots \dots \text{Pd}_{n-1} \text{OH} \times \text{H} \cdot \text{Pd}_n)}{(\text{Protein})} = K, \text{ or (1)}$$

$$\frac{(\text{Pd})^n}{(\text{Protein})} = K \quad (2)$$

If the initial concentration of protein be denoted by C , the degree of hydrolysis by h , equation (2) may be written:

$$\frac{(h \cdot C)^n}{(1-h)C} = K \quad (3)$$

$$\frac{h^n C^{n-1}}{(1-h)} = K \quad (4)$$

$$C^{n-1} = K \frac{(1-h)}{h^n} \quad (5)$$

The first deduction from equation (5) is that the degree of hydrolysis, h , is dependent upon the initial concentration C of the protein in solution, and that, as it increases, h decreases; and, therefore, the possibility for synthesis increases. If the initial concentration is high, the degree of hydrolysis is small, and at equilibrium relatively little protein is hydrolyzed; this is especially marked for proteins, where n is high. If there is no protein at the outset of the

reaction, as is the case when a solution of the products of digestion is concentrated down, then sufficient protein is synthesized to provide the amount required to satisfy the equilibrium equation for the degree of hydrolysis existing at that concentration of substances in solution.

From equation (5) it is seen that as C increases in arithmetrical progression, as it does under experimental conditions, h decreases in geometrical progression, and hence the tendency for synthesis, which is a function of the reciprocal of h , increases in geometrical progression. Decreasing C in arithmetrical progression, the reverse holds true. Therefore, where n is high, in concentrated solutions, the tendency for synthesis is great and in dilute solutions the tendency for hydrolysis is great. The difficulties encountered in accounting on simple physicochemical grounds for synthesis *in vitro*, or in the tissues, thus vanish when the equilibrium equation for the hydrolysis of protein is properly stated.

SUMMARY.

1. Certain theoretical considerations are pointed out, which lead to the conclusion that the synthesis of protein from the products of hydrolysis should be possible *in vitro*, without difficulty, and without the interposition of any special mechanism for effecting abnormally high concentrations of products.

2. Experimental confirmation of this conclusion is demonstrated with pepsin in a concentrated solution of the products of peptic hydrolysis of egg albumin. Under optimal conditions 39 per cent of the nitrogen of the original digest was found in the synthesized protein.

3. Evidence is supplied that the substance synthesized is of the order of complexity of native protein.

4. The reaction is a reversible one, in that a linkage is synthesized in concentrated solution, which is hydrolyzed in dilute solution.

5. A phenomenon is described which may account for the solubility properties of the protein synthesized.

6. The products of the peptic hydrolysis of gelatin were found to be incapable of synthesis by means of pepsin. The presence

of tyrosine and tryptophane, or synthesizable albumin radicles, also failed to effect this synthesis.

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ANALYTICAL METHODS AND OBSERVATIONS ON THE ORGANIC PHOSPHORUS OF THE URINE.

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It is rather well known that our knowledge of the phosphorus occurring in organic combination in the urine is from many stand-points an uncertain factor, although now accepted that such a fraction of the total urinary phosphorus occurs, and most probably as a glyceryl phosphate (1).

The literature on this subject is reviewed and summarized in the papers of Kondo (2), Mathison (3), and Feigl (4).

The analytical methods for urinary organic phosphorus, which have been employed heretofore, have given variable results and it has become evident that better procedures would be desirable. In the following paragraphs we report work done in that direction, together with results obtained with a number of human urines.

Analytical Methods for Organic Phosphorus in Urine.

The analytical methods may be divided into two classes: (a) those in which the total and inorganic phosphorus are each separately determined, their difference representing the organic fraction; and (b) those in which the inorganic phosphates are removed by precipitation and the organic phosphorus determined separately in the filtrate.

In the first class we have the difficulty that the errors of the method fall on the organic fraction and since this is comparatively small, it has hardly been possible to obtain anything but indicative results in that way. LeClerc and Cook (5), F. G. Benedict

(6), and Pasqualis (7) do not accept any organic phosphorus in the urine in their work based on such determinations. The figures of LeClerc and Cook, however, show 3 to 4 per cent of organic phosphorus if no errors in the method are accepted.

Marcuse (8), Zuelzer (9), Oertel (10), and others, using more or less modified procedures as to the precipitating reagents and ways of conversion of organic phosphoric acid to inorganic phosphate, report definite amounts and percentages of organic phosphorus as reliable figures.

Taylor and Miller (11) state that it is not permissible to estimate organic phosphorus by difference.

In the second class—separate determination after removal of inorganic phosphates by precipitation—we find data which doubtless are closer to the true values. However, precipitating reagents have been used which do not completely remove inorganic phosphates, or which retain organic phosphorus compounds in the precipitate. The reagents are indicated in this paper.

Especially in view of the Bell and Doisy colorimetric method (12) it does not now seem permissible to determine organic phosphorus by difference. We have therefore turned our attention to the accurate removal of inorganic phosphates and subsequent determination of the residual phosphorus.

As precipitants for inorganic phosphates we find that the following have been employed: (a) barium hydroxide, (b) barium chloride, (c) calcium chloride, (d) ferric chloride, (e) magnesium citrate, and (f) magnesia mixture.

In our work we find the following concerning each.

Barium Hydroxide.—This does not completely remove inorganic phosphate from urine or phosphate solutions when made alkaline to phenolphthalein. It requires a large excess over the calculated amount.

Further, the barium precipitate in some way retains (occludes) organic phosphorus compounds to a considerable extent.

Barium Chloride.—The conditions for precipitation and occlusion would be the same as for barium hydroxide.

Calcium Chloride.—Inorganic phosphates are not completely precipitated with excess of calcium chloride in ammoniacal solution. We have obtained results which were 50 per cent higher than those by magnesia mixture or by magnesium citrate pre-

cipitation. The Bell and Doisy reagents or Scott's reagent give strongly positive tests for inorganic phosphates in the filtrate.

Ferric Chloride.—According to Scott (13) this reagent completely precipitates soluble phosphates in acetic acid-sodium acetate solution as FePO_4 . We find that the precipitation is not complete. Besides finding inorganic phosphate in the filtrate, the organic phosphorus values for urines are usually about 2.5 times higher than by precipitation with either magnesia mixture or magnesium citrate.

Magnesium Citrate.—We were able to obtain results by magnesium citrate precipitation (14) which from all considerations seemed correct. Especially they were substantially the same as those of magnesia mixture precipitation. The inorganic phosphates are completely precipitated. Mathison (14) reports favorably on this method and Bell and Doisy find that it gives higher but probably more accurate results than the barium precipitation. The process, however, is unsatisfactory for the following reasons: (a) difficulty of digestion with sulfuric and nitric acids due to the citrate radicle; (b) difficulty of determining complete removal of inorganic phosphates; (c) length of time for complete precipitation; and (d) the magnesium citrate mixture is inconvenient to prepare.

Magnesia Mixture.—(Composition given under "Analytical method used in this work.")

This well known reagent for the precipitation of inorganic phosphates has not been recommended by all workers. Mathison found that the mixture precipitates a portion of the organic phosphorus. In our experiments with sodium glycerophosphate we do not find that to be the case.

The possibility of the conversion of organic phosphorus compounds to inorganic by the ammonia of the magnesia mixture during the time of standing in precipitating inorganic phosphates was suggested by Mathison, who kept urine in ammonia at 37°C . and found much decomposition during 7 days. He concluded that it was important not to leave the urine standing too long after adding magnesium citrate mixture (which is similar to magnesia mixture so far as the ammonia content is concerned). We therefore determined the extent of such decomposition under the conditions of magnesia mixture precipitation; that is, 100 cc.

of urine containing 25 cc. of concentrated ammonia water were allowed to stand for 7 days and the organic phosphorus was determined at the beginning and at the end of the period. The following were the results.

Fresh urine.....	1.090 mg. organic P per 100 cc.
Same urine + ammonia, after 7 days ¹ ..	1.077 " " " " 100 "
Loss.....	about 1.2 per cent.

This loss is within experimental error of the colorimetric method. We have further confirmed the very slow change in value for organic phosphorus in urines.

Many allow 24 hours for magnesia mixture precipitation. Olsen (15) designates at least 6 hours. We found the following values for a urine.

Determination No.	Time of standing with magnesia mixture.	Organic P.
	<i>hrs.</i>	<i>mg. per 100 cc.</i>
1	2	0.430
2	3	0.428
3	6	0.412
4	24	0.436

Although 2 hours are sufficient, we have, as a matter of safety with all urines, allowed 6 hours standing.

Analytical Method Used in This Work.

In our application of the Bell and Doisy colorimetric method we have encountered various difficulties, but we have surmounted all but one of them by suitably modifying the technique. Bell and Doisy made only several determinations of organic phosphorus in urine and apparently did not attempt to check up the accuracy of the procedure except on blood.

The inorganic phosphates are precipitated with magnesia mixture and the filtrate from the precipitation is digested in a Pyrex test-tube (200 × 25 mm.) with sulfuric and nitric acids as in the Bell and Doisy procedure except that we use tubes graduated at 25 cc., and we also add copper sulfate. This hastens the decom-

¹ Corrected for dilution with ammonia.

position, overcoming long digestion which causes loss of phosphorus by volatilization. The copper sulfate also acts as the indicator in the subsequent neutralization with ammonia, giving the usual deep color near the neutral point. In subsequent acid solution, however, the copper gives only a trace of color.

After diluting the cooled mixture we neutralize with phosphorus-free (redistilled) ammonia water. Upon reacidifying we are thus enabled to have the same degree of acidity in the unknown and the standard. This is convenient and necessary for accurate results.

The molybdic acid, hydroquinone, and carbonate-sulfite solutions then added are prepared as recommended by Bell and Doisy.

TABLE I.

Recovery Determinations of Phosphorus on Urines with Added Phosphate.

Urine No.	Added P.	Total expected.	Total found.	Loss of added P.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	0.025	0.0748	0.0725	9.2
2	0.025	0.0737	0.0721	6.4
3	0.025	0.0664	0.0636	11.2
4	0.025	0.0664	0.0633	12.4
5	0.025	0.0664	0.0644	8.0
6	0.025	0.0651	0.0612	15.6
7	0.025	0.0501	0.0469	12.8
8	0.025	0.0630	0.0591	15.6
9	0.050	0.0880	0.0820	12.0
10	0.100	0.1487	0.1351	13.6
Average.....				11.7

In place of transferring the mixture at any stage, it is kept in the same Pyrex tube and finally made up to the 25 cc. mark and read in the colorimeter.

Our main difficulty has been the loss of phosphorus in digestion with the acids. A loss has already been pointed out by Hillebrand and Lundell (16) and lately by Baumann (17) who finds that although phosphoric acid when heated alone does not volatilize until about 260°C. is reached, it volatilizes slowly in the vapors of sulfuric acid at 180–200°C. In the Bell and Doisy method he found losses of from 12 to 15 per cent. Baumann

also believes that there is some conversion of *o*- to pyro-phosphoric acid. We have not found this difficulty, nor is there any *m*-phosphoric acid formed in our process.

Our losses by volatilization are shown in Table I. They are about the same as those found by Baumann. We have not yet found a way to overcome all of this loss and the results reported in the experimental work of this paper are a little low. We were careful to guard against too high a temperature in oxidizing and we do not believe our results are more than several per cent too low, although in our quantitative tests for per cent loss—made under more drastic heating—they averaged 11.7 per cent, maximum 15.6 per cent.

We have attempted to apply Baumann's process of oxidizing blood and tissue extracts with sulfuric acid and 30 per cent hydrogen peroxide (superoxol, Merck)² to the oxidation of urine, but without success. The amount of organic matter is too large.

We have not changed from the Bell and Doisy method to the modification of Briggs (18) because the green color of the latter is not so intense. In spite of fading more rapidly, the blue color development of Bell and Doisy seemed preferable for this work.

Special Reagents Required.

Magnesia Mixture.—This is made by dissolving 55 gm. of crystallized magnesium chloride and 70 gm. of ammonium chloride in water and adding 300 cc. of dilute, or 88 cc. of concentrated, ammonia water, sp. gr. 0.90, and diluting to 1 liter. After standing for several days the solution is filtered as required for use.

Redistilled Ammonia.—Concentrated ammonia water is distilled and diluted with water, 1:1. The distillation can be done in any ordinary laboratory distillation apparatus, in a fume cupboard. Considerable phosphorus was found in c.p. ammonia water as usually purchased. Sodium hydroxide or sodium carbonate is not so easily freed of phosphorus compounds.

Copper Sulfate.—10 per cent solution.

The following are prepared according to the directions of Bell and Doisy (12).

² The reagent complied to specifications as to H_2O_2 .

Standard Phosphate Solutions.

Molybdic Acid Solution.—This solution gathers phosphorus from ordinary glass reagent bottles in the course of a few weeks. It is best to keep it in paraffined containers in order to obviate this.

*Hydroquinone Solution.**Carbonate-Sulfite Solution.*

TABLE II.

Data Showing Rate of Disappearance of Organic Phosphorus in Urines on Standing.

The urines were preserved with chloroform and were analyzed on the days indicated.

Urine No.	Day.	Organic P.	Loss.
		<i>mg. per 100 cc.</i>	<i>per cent</i>
1	1st	0.647	
	4th	0.658	None.
	9th	0.636	1.7
	16th	0.633	2.2
	46th	0.628	3.0
2	1st	0.704	
	4th	0.708	None.
	9th	0.691	1.8
	16th	0.591	16.1
	46th	0.588	16.5
3	1st	0.487	
	4th	0.469	3.7
	9th	0.452	7.2
	16th	0.382	21.6
	46th	0.370	24.0

Procedure.

25 cc. of urine and 7 cc. of magnesia mixture are transferred to a 100 cc. Erlenmeyer flask, mixed, allowed to stand 6 to 18 hours, and 5 cc. (10 cc. of dilute urine) of the filtrate are transferred to a 200 × 25 mm. Pyrex test-tube graduated at 25 cc. 12 drops of concentrated sulfuric acid, 2 drops of 10 per cent copper sulfate, and 2 or 3 pieces of silica ware are added. The tube is clamped upright over a micro burner and the contents are carefully boiled down to about 2 cc. 1 cc. of concentrated nitric acid is added and the heating is continued until the con-

TABLE III.
Rate of Excretion of Organic Phosphorus in Urines of Twelve Normal Persons.

Subject No.		6-8 a.m.	8-10 a.m.	10 a.m.- 12 n.	12 n.- 2 p.m.	2-4 p.m.	4-6 p.m.	6-8 p.m.	8-10 p.m.	10 p.m.- 12 n.	12 m.- 2 a.m.	2-4 a.m.	4-6 a.m.	Total 24 hrs.	Weight of subject.		Organic P per kg. in 24 hrs.
															kg.	mg.	
1	V.* P.	220 0.803	70 0.364	270 0.698	683 1.434	260 0.671	239 0.786	677 1.319	211 0.752	109 0.731	445 0.943	186 0.871	213 0.827	3,584 10,199	80.9	0.126	
2	V. P.	51 0.622	74 0.628	77 0.720	44 0.652	64 0.804	86 0.891	121 1.073	136 1.008	98 0.678	95 0.737	80 0.773	50 0.595	976 9,181	70.0	0.131	
3	V. P.	48 0.481	87 0.679	257 0.759	192 0.922	103 0.887	186 0.936	106 1.052	153 0.991	88 0.717	77 0.672	114 0.695	120 0.737	1,531 9,528	63.2	0.150	
4	V. P.	26 0.206	107 0.585	228 0.529	265 0.500	273 0.558	135 0.649	102 0.500	111 0.599	105 0.696	58 0.474	38 0.349	51 0.436	1,499 6,081	68.2	0.089	
5	V. P.	29 0.381	34 0.403	49 0.379	107 0.601	294 0.628	213 0.433	270 0.574	275 0.571	68 0.589	34 0.447	26 0.366	20 0.341	1,419 5,713	58.2	0.098	
6	V. P.	87 0.794	92 0.737	69 0.705	45 0.392	275 1.099	80 0.560	100 0.923	143 1.098	190 1.150	63 0.478	81 0.901	39 0.526	1,264 9,363	72.7	0.128	
7	V. P.	53 0.704	41 0.645	49 0.653	92 1.098	203 1.341	203 1.287	145 1.292	110 1.427	98 1.308	180 1.622	54 0.659	45 0.679	1,273 12,715	67.7	0.187	
8	V. P.	65 1.148	150 0.808	29 0.320	46 0.506	162 1.294	78 0.837	85 1.005	68 0.948	37 0.605	34 0.606	115 0.830	32 0.411	901 9,318	70.9	0.131	

9	V.	58	98	175	83	95	79	102	81	118	47	72	58	1,066	79.5	0.131
	P.	0.563	0.795	0.727	0.738	1.138	0.988	1.405	0.772	1.256	0.555	0.838	0.671	10.446		
10	V.	64	76	68	104	91	81	77	66	53	41	42	59	832	70.9	0.141
	P.	0.736	0.619	0.683	0.789	0.879	0.889	0.965	1.039	1.009	0.796	0.846	0.781	10.031		
11	V.	40	27	62	71	191	263	63	167	68	40	67	48	1,107	66.4	0.146
	P.	0.652	0.478	0.911	0.735	0.944	0.905	0.624	1.176	0.923	0.649	1.011	0.697	9.705		
12	V.	44	52	44	36	37	39	55	74	75	58	47	55	616	70.0	0.110
	P.	0.705	0.526	0.463	0.468	0.583	0.580	0.605	0.830	0.805	0.734	0.691	0.742	7.732		
Average.	V.	65.4	75.7	114.7	147.3	170.7	140.2	158.6	133.0	92.2	97.7	76.8	65.8	1,339	69.9	
	P.	0.650	0.606	0.629	0.736	0.902	0.812	0.945	0.934	0.872	0.726	0.736	0.620	9.168		0.131

* V represents volume in cubic centimeters; P, phosphorus in milligrams.

tents are clear and only colored by the copper. If necessary a little more nitric acid may be added. After permitting the mixture to cool somewhat, 5 cc. of water are added, rinsing down the sides of the tube. The solution is then heated to boiling to insure that all of the contents are dissolved, and is then neutralized to a deep blue color with redistilled ammonia water. It usually requires from 3 to 5 cc. of the ammonia.

The standard is then prepared by transferring 5 cc. of the standard phosphate solution (0.025 mg. of P) and 2 drops of 10 per cent copper sulfate solution to a similar graduated tube and adding water to make approximately the same volume as the unknown.

To both tubes are then added 5 or 6 drops of concentrated sulfuric acid, 1 cc. of molybdic acid solution, and 2 cc. of hydroquinone.

After 5 minutes 10 cc. of the carbonate-sulfite solution are added and the contents made up to the 25 cc. mark, well mixed, and read in the colorimeter, setting the standard at 30 mm.

Calculation.

If 5 cc. of the urine filtrate (representing 3.91 cc. of urine) and 5 cc. of the standard (representing 0.025 mg. of P), are taken, with the standard set at 30 mm. the calculation is

$$\frac{19.2}{\text{reading}} = \text{mg. organic P per 100 cc. original urine}$$

As stated by Bell and Doisy, urines high in phosphorus may be read against a stronger standard. But it is better for most urines to use the smaller standard, since the smaller the amount of urine the easier the digestion.

EXPERIMENTAL.

Table II shows analytical data on the losses of organic phosphorus in urines on standing at room temperature. These urines were slightly acid and were preserved with chloroform. They were analyzed within 2 days after voiding.

Table III contains data for the organic phosphorus excretion of twelve medical students (males) for 24 hours, in 2 hour periods.

TABLE IV.

24 Hour Output of Organic Phosphorus of Old People and Children.

Subject.	Date.	Urine volume.	Organic P.	Age.	Sex.	Pathology.
	<i>1923-24</i>	<i>cc.</i>	<i>mg.</i>			
1	Dec. 29	1,520	8.32	63	M.	Arteriosclerosis.
	" 30	1,840	15.89			
	" 31	1,700	18.27			
	Jan. 2	1,820	8.65			
Average.....			12.78			
2	Dec. 30	1,240	6.67	60	F.	Pernicious anemia (?)
	" 31	1,060	6.36			
	Jan. 1	1,340	11.73			
	" 2	620	4.96			
	" 4	800	9.34			
Average.....			7.81			
3	Jan. 17	760	6.51	73	M.	Fracture.
	" 18	360	4.53			
	" 19	460	2.81			
Average.....			4.95			
4	Jan. 16	420	4.10	49	F.	Laparotomy.
	" 17	460	3.75			
	" 18	400	4.37			
	" 19	720	8.04			
	" 20	660	5.68			
Average.....			5.19			
5	Jan. 14	700	7.02	60	F.	Fracture.
	" 15	1,320	9.24			
	" 16	900	10.52			
	" 17	440	3.56			
	" 18	620	5.81			
	" 19	420	4.97			
Average.....			6.85			
S. F.	May 16	1,720	15.48	13	M.	Frozen toe.
G. V.	" 16	1,760	5.06	10	F.	Diabetes.
M. M.	" 16	1,000	8.00	10	"	Lues (+ +).
H. K.	" 16	1,800	7.42	8	"	Spinal tuberculosis.
T. S.	" 16	1,320	9.37	10	M.	Tonsil trouble.
Average.....			9.07			

The urine volumes and subject weights are given and the averages of organic phosphorus and volumes are shown. The students were cautioned to observe their usual diets and were required to make written reports on the food eaten and the time of their meals. These data present nothing unusual and will not be further mentioned except to state that usually three meals a day, at 8 a.m. and 1 and 6 p.m., was the rule. The subjects were instructed to drink water *ad libitum* according to their

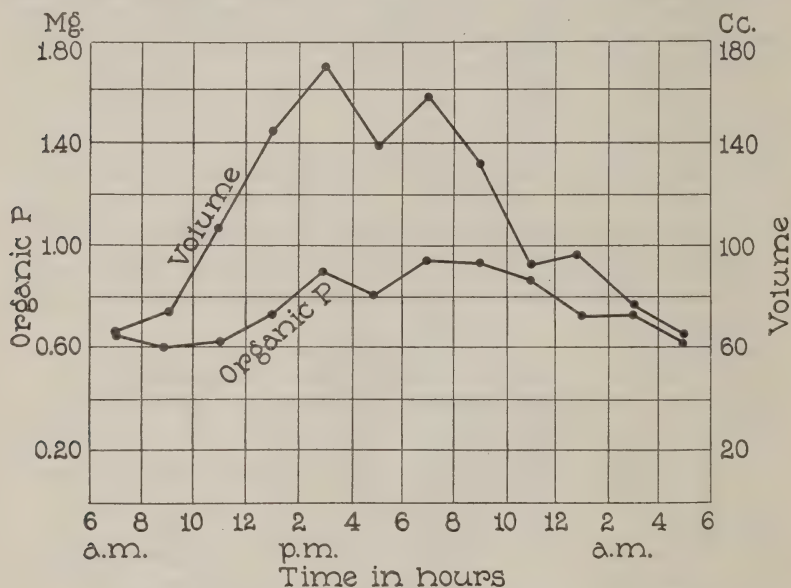


FIG. 1. The relation between organic phosphorus excretion and volume of urine. The curves are the averages for twelve normal persons. (From Table III.)

usual habits. Subject 1, however, reported that he drank more water during the period than was usual for him.

Table IV shows some typical cases of the daily variations of organic phosphorus excretion for old persons and children. The subjects were hospital cases with the pathology indicated. The diets were not taken into account, except that nothing unusual was noted for such cases.

Fig. 1 presents the curves of organic phosphorus excretion and urine volume from the data of Table III.

SUMMARY AND CONCLUSIONS.

The analytical methods for organic phosphorus are considered. The method chosen for this work was the precipitation and removal of inorganic phosphates with magnesia mixture and the subsequent determination of organic phosphorus in the filtrate by a modification of the recent colorimetric method of Bell and Doisy. Our procedure gives somewhat low results on account of volatilization of phosphorus when heating with sulfuric and nitric acids to destroy organic matter.

The organic phosphorus excretion in normal man fluctuates considerably during the day and also at night. A change of 100 per cent in successive 2 hour periods is often found to occur, and even larger changes are shown in Table III. One cannot predict the excretion for any single individual.

The output per kilo of body weight is not constant. In twelve normal persons the lowest value is seen to be 0.089 mg., the highest 0.187 mg., with an average of 0.131 mg. per kilo in 24 hours.

As represented in Fig. 1 the urine volume can be only a minor factor in affecting the organic phosphorus excretion.

The excretions of organic phosphorus which we found are considerably lower than those found by others, except those reported by Mandel and Oertel (19).

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INTESTINAL CHEMISTRY.

I. THE ESTIMATION OF INTESTINAL REDUCTIONS.

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College of Medicine, Chicago.)*

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The intestines of men and animals are the seat of powerful reduction processes. The biliverdin and bilirubin of the bile are here reduced to urobilin and still further to the colorless urobilinogen. Bismuth salts are reduced to the suboxide and ferric salts to the ferrous condition. Pigments such as methylene blue and litmus are decolorized. This strongly reducing character of the intestinal contents cannot be without influence on some of the many chemical reactions occurring within the intestines. The oxygen supply must be of particular importance for the bacteriology of the intestine, the removal of oxygen by reducing substances presumably favoring the growth of obligate anaerobes among which are organisms of putrefaction responsible for the formation of those products which are considered chiefly responsible for the toxemias of intestinal origin. Furthermore, it is reasonable to suppose that in as far as the reducing substances, found in the intestine, are of bacterial origin, their estimation would give an index of certain types of bacterial action in the intestine.

No attempt seems to have been made, however, to obtain a quantitative measure of the extent to which reduction processes may occur in the gastrointestinal tract under varying conditions. Certain reducing substances such as formic acid, hydrogen, and hydrogen sulfide, are known to be formed in the intestine by bacterial action. Reducing substances such as simple sugars may also be formed in the normal processes of digestion. Most of these reducing substances, however, are soluble and readily absorbed

so that the amounts present in the feces may be very incorrect indices of the extent of their formation in the bowel.

In order, therefore, to determine the extent and variations in the formation of reducing substances in the intestine, and thus to obtain information as to their significance, a new type of procedure seems necessary. Such a method may consist in the feeding of a reducible substance and the determination of the extent of its reduction by fecal analyses. The substance fed should not be absorbed from the intestine in significant amounts as fecal analyses would then give incomplete data. For the same reason the substance should be one which is not destroyed by any processes, either digestive or bacterial, occurring in the intestine unless it be certain that this destruction be due entirely to reduction. The substance should be non-toxic when given in amounts necessary to insure incomplete reduction and should produce as little disturbance as possible of intestinal digestion, of intestinal motility, and of the bacterial processes occurring in the intestine. The substance should further be one readily determined in the feces which means that it must be readily separated from the complex mixture of interfering substances found in feces and must not be reoxidized so easily as to require elaborate technical provisions to prevent this. It is further desirable that the substance be determinable in minute amounts in order that manipulation may be facilitated by the small amounts of feces required and particularly in order that experiments may be carried out on small animals such as the rat. It is likewise extremely desirable that the reducible substance employed be one which can be reoxidized to form the original compound in order that we may be able to show that the apparent disappearance of the substance was due solely to reduction and especially because it is then possible to determine both the amounts reduced and unreduced and calculate directly the percentage reduction. The tremendous advantage here lies in the fact that the percentage reduction may then be determined without the difficult and laborious separation and mixing of all feces containing the test substance with the necessary storage of feces for 2 or 3 days during which time it would further be difficult to prevent reoxidation which would vitiate the results. As a clinical procedure this has the further advantage of making it unnecessary to weigh the material for analysis, a considerable

advantage where many determinations are to be made particularly as weights of a moist substance such as feces are usually obtained by difference and thus require two weighings for each sample.

The complex requirements for a test of this character are very well met by the use of ferric oxide as a test substance and the procedure outlined below which can be carried out by an unskilled worker in a few minutes. From the standpoint of the chemical determination nothing simpler or more accurate could be desired. The question as to whether the ferric oxide used may have any undesirable effects or produce any alterations in intestinal activities cannot be answered with the same ease. Obviously a pure oxide (especially one free from arsenic) must be used. Ferric oxide is a very insoluble substance hardly at all absorbed from the intestine and hence should produce no symptoms due to systemic action. Soluble ferric salts possess some astringent action presumably much greater than that of insoluble ferric oxide and yet are given therapeutically over considerable periods of time. Iron oxide has further been given to pregnant sows in doses of 40 gm. per day over periods of several weeks with advantage to the offspring.¹ No detrimental effects have been noted in our experiments. It has, however, seemed desirable to give with the iron oxide an equal weight of powdered agar-agar to counteract any constipating tendency of the iron and not to continue iron administration over longer periods of time than necessary.

The method employed for the study of the reduction processes in the intestine of the albino rat is given below. The method with slight modifications (to be presented in another paper) is equally applicable to studies on the human subject.

Method.

The animals under investigation are given any desired diet with which the iron salt used is incorporated to make a uniform mixture. A mixture with 1 per cent of hydrated ferric oxide powder (completely soluble in 100 parts of 12 per cent hydrochloric acid in about 30 seconds on the boiling water bath) or one-fourth this amount of a coarser ferric oxide (dissolving in

¹ McGowan, J. P., and Crichton, A., *Biochem. J.*, 1924, xviii, 265.

about 2 minutes) may be used. The same preparation should be used throughout a given series of experiments.

After 2 days on the experimental diet specimens of feces are collected. In the case of the rat these may be obtained by digital pressure on the lower bowel.

About 0.2 gm. of feces (not weighed) is rubbed up with 10 cc. of dilute HCl (1:2) and the mixture heated in a test-tube of about 18 mm. diameter on a boiling water bath for $\frac{1}{2}$ minute (2 minutes if the coarser iron oxide is used). If biliary or other pigment be present in appreciable amount 0.5 gm. of acid-extracted blood charcoal should be added before heating. This is usually not necessary in studies on rats, but is required in the examination of human feces. The tubes are placed in cool water for about $\frac{1}{2}$ minute and then filtered preferably within $\frac{1}{2}$ hour. 2 or 3 cc. aliquots of the filtrate are measured into each of two 25 cc. graduated cylinders. To one add enough 0.1 N potassium permanganate solution to give a pink color lasting for about 1 minute (1 or 2 cc. are usually required). This oxidizes the ferrous iron to the ferric condition. Then to each cylinder add 2 or 3 cc. of a 1 per cent solution of potassium thiocyanate and make each to volume (usually 7 to 10 cc.). Compare the two solutions obtained in a colorimeter. Divide the lower by the higher reading and subtract from 100. This gives the percentage of ferric oxide reduced. A determination requires only a few minutes and a large number of determinations can conveniently be run through together.

SUMMARY.

The fact that ferric oxide when ingested with foods is reduced in the intestine to the ferrous form and that both forms are readily estimated in the feces is made the basis of a simple method for obtaining a quantitative index of reduction processes in the intestine. This method furnishes a simple index of intestinal putrefaction.

INTESTINAL CHEMISTRY.

II. INTESTINAL REDUCTIONS AS MEASURES OF INTESTINAL PUTREFACTION, WITH SOME OBSERVATIONS ON THE INFLUENCE OF DIET.

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In a preceding paper (1) a simple method is described for estimating reduction processes in the intestine by a determination of the amount of unreduced ferric oxide found in the feces after ingestion of a definite amount of this substance. Further observations made it apparent that this method was of value in the study of intestinal putrefactive processes and data derived from experiments upon albino rats supporting this view are given in the present paper. Experiments on men and on other animals will be reported in a later communication.

More satisfactory chemical indices of intestinal putrefaction than are at present available are much needed. How unsatisfactory are present methods for the study of the extremely important problems involved becomes apparent when we consider the status of the indican determination which on a recent re-examination of methods was found to be the most reliable of these (2). In the first place there is no satisfactory method for the determination of indican. Secondly, an unknown amount of the indole produced in the intestine is destroyed within the body and the amount so destroyed is probably not constant at least for different individuals. Nor is a constant amount of the indole formed absorbed from the intestines. Indole is formed from tryptophane and is dependent upon the amount of this amino acid in the diet. Further, in some severe forms of intestinal putrefaction little indole may be produced. Phenol and conjugated sulfate determinations have not proven more satisfactory.

A more accurate and direct index of intestinal putrefaction is, therefore, very desirable.

The following experiments were carried out on albino rats of about 100 gm. in weight. The animals were given the diets indicated which contained usually 1 per cent of hydrated ferric oxide and an equal amount of powdered agar-agar to overcome any tendency to constipation. After 2 days on a given diet specimens of feces were collected by digital pressure on the lower intestine, material being thus obtained which had not been subjected to reoxidation in the air. This procedure offers no difficulty in the rat. Specimens were collected on several successive days and analyzed for comparative amounts of reduced and unreduced iron as described in a previous paper (1). Results are expressed as percentage of iron reduced.

Reduction in Different Parts of the Gastrointestinal Tract.

The earliest experiments showed that iron oxide to the amount of 1 per cent of the diet might be reduced in the intestine to the extent of 80 per cent or more. This reduction might conceivably be due in part or whole to reducing substances in the diet as ingested (*i.e.* raw meat as suggested by Herter (3) or glucose); to reducing substances formed during digestion (as glucose from starch); to reducing substances such as formic acid formed by organisms in the small intestine acting particularly on carbohydrate material (the process being commonly called intestinal fermentation); to products formed in the rat cecum as the result of cellulose digestion; or finally, to products of the action of the bacteria of the lower intestine on incompletely digested protein (the process designated as intestinal putrefaction). To estimate the relative importance of these factors, studies were made of the extent of reduction of iron in different parts of the gastrointestinal tract and on different diets. Rats were given the test diets for several days, then killed, and analyses made of the material at various levels of the tract. In Table I are given results of experiments of this type.

These results show that no reduction took place in the stomach and practically none in the upper small intestine. This was true even though marked intestinal fermentation was found to exist in the case of the animals on the flour-lactose diet. Reduction

due to sugars or to foods such as raw meat or in general to any products of digestion or of intestinal fermentation could, therefore, represent but a small part of the total reduction noted. In the lower small intestine where putrefaction begins reduction also became noticeable. By far the greater part of the reduction took place, however, in the cecum and large intestine where intestinal putrefaction predominates. That products of cellulose digestion are not responsible will be clear from the experiment on meat and from other results given in succeeding tables as well as from the fact that human subjects give reductions of the same order of magnitude although cellulose is not extensively digested. For these reasons also the results of experiments on rats become more generally applicable to man. The fact is,

TABLE I.

Intestinal Reduction in Different Parts of the Gastrointestinal Tract of the Albino Rat.

Organ.	Diets.		
	Raw meat.	Flour-meat.	Flour-lactose.
Stomach.....	0	0	0
Jejunum.....	0	5	0
Ileum.....	0	10	10
Cecum.....	65	68	33
Colon.....	44	64	25

however, also brought out that the rat may not be an ideal animal to use in an attempt at any accurate standardization of foodstuffs for the reduction brought about by their presence in the diet because of the fact that the food residues passing through the cecum of this animal do not always remain in this organ for exactly the same length of time. Some of the material may be side-tracked for a greater length of time than some other portion and this should be less true in animals in which the cecum plays a minor rôle. However, where fine distinctions are not necessary the rat is the most convenient animal to employ. The iron oxides give definite colors to the intestinal contents so that the lapse of time before they appear in the feces is a measure of intestinal motility. Intestinal motility is of great significance for intestinal putrefaction and must always be considered in comparative studies.

Influence of the Character of Protein in the Diet.

Through the work of many investigators we possess a certain amount of knowledge of the influence of different dietaries on the intestinal flora and on the extent of intestinal putrefaction. Studies of intestinal reductions on similar diets should, therefore, enable us to correlate the latter processes with the former. Furthermore, practical methods of decreasing either intestinal putrefaction or the reduction processes associated therewith must depend upon the diet. Different food factors were, therefore, studied. Because most common food factors are mixtures it was thought best in order to prevent confusion to first test out pure proteins, fats, and carbohydrates. The following proteins

TABLE II.

Influence of Proteins in the Diet on Intestinal Reduction.

Starch-protein diets. Protein 20 per cent; agar 1 per cent.

Protein.	Reduction.
	<i>per cent</i>
Egg albumin powder.....	71
Meat (cooked) powdered.....	63
Casein powdered.....	19
Peptone (Witte).....	43
Gelatin powdered.....	40
Egg albumin and 20 per cent butter.....	68

were used: egg albumin, meat powder, peptone, gelatin, and casein. The findings are given in Table II. Egg albumin showed the highest reduction percentage (71), and casein the lowest (19). These values were obtained on protein-starch diets with protein equivalent to 20 per cent. That casein is of these proteins the least liable to putrefaction in the intestine is supported by all available data and it certainly gives low reduction values. In the case of gelatin indican estimations completely fail to give any measure of intestinal putrefaction as gelatin contains none of the tryptophane which is the precursor of indican in the body.

The high reduction with egg albumin diets may partly be due to the high sulfur (cystine) content of this protein giving rise to the formation of larger amounts of the powerfully reducing substance, hydrogen sulfide. The exact importance of hydrogen

sulfide in this connection remains to be determined, but it is undoubtedly considerable. No close relation exists in bacterial cultures outside the body between reducing power and hydrogen sulfide formation according to the reports of bacteriologists. We have found a certain degree, but not a high degree, of parallelism to exist between reduction and fecal sulfide as determined in connection with certain of these experiments. It must be realized, however, that all sulfide formed in the intestine is not excreted in the feces, some being presumably absorbed and some oxidized, whereas practically none of the iron is absorbed. If reduction were largely due to sulfide the reduction test should, therefore, still be superior to direct sulfide determination as an index of intestinal putrefaction. Hydrogen sulfide appears to be solely a product of putrefaction in the intestine and is an important substance. It is a product readily formed in the intestine and possesses a high degree of toxicity. 5 volumes in 10,000 will produce death (4) by inhalation; the power of the body to oxidize the sulfide being apparently quite limited. The authors mentioned hold that the action is not on the hemoglobin but upon nerve cells. Many of the symptoms of sulfide poisoning are similar to those of intestinal toxemia (headache, dizziness, depression, etc.).

McGowan and Crichton (5) have made the interesting observation that pregnant sows kept on a diet which among other things is low in iron give birth to anemic offspring, this influence of the diet being counteracted by giving 40 gm. of ferric oxide per day. This ferric oxide might act by decomposing the toxic hydrogen sulfide formed from the diet. On the other hand, certain vitamins are known to be destroyed by reduction (6), and the same is probably true of other substances found in the intestine.

Hydrogen sulfide may be determined in conjunction with the reduction test by taking an aliquot (2 cc.) of the filtrate obtained and aspirating into an equal volume of an alkaline lead acetate-gelatin solution (containing gelatin 0.5 per cent, NaOH 0.5 per cent, and lead acetate 0.05 per cent) and comparing with suspensions of lead sulfide obtained by treating in the same way known solutions of hydrogen sulfide standardized by iodine titration. We have used the gelatin to stabilize the lead sulfide sus-

pension and thus made practicable this method by means of which very minute amounts of sulfide such as found in 0.1 gm. of rat feces may be estimated.

Influence of Carbohydrates and Fat in the Diet.

Carbohydrate diets were studied in some detail. In culture media soluble carbohydrates, such as glucose, decrease putrefaction first by furnishing a readily available source of energy preferred for this purpose to protein by many bacteria the amount of protein decomposed being, therefore, decreased and also because the acid products formed from glucose produce a medium less favorable to the growth of putrefactive organisms. Glucose, however, when ingested is absorbed so quickly that it has little opportunity to produce these effects in the large bowel and is, therefore, not nearly so useful as lactose and dextrin which, when given in fairly large amounts, in part reach the colon and do modify the intestinal flora profoundly to an aciduric type, *Bacillus acidophilus* predominating. Attempts made by using the indican determination to show a decreased intestinal putrefaction on these diets or on diets containing also cultures of *Bacillus acidophilus* have been inconclusive, this in spite of the undoubted therapeutic results obtained. That the chemistry of the intestine is profoundly altered by lactose administration is quite apparent from the following results.

Starch, sucrose, glucose, fructose, maltose, lactose, and dextrin were studied. These made up in different experiments 20, 30, and 75 per cent of the diets used. As a protein egg albumin was given to the extent of 20 per cent. In another series of experiments the carbohydrates, starch, sucrose, and lactose, were added to a standard bread-meat diet (dry bread 75, meat powder 20, agar 2, ferric oxide 1) to make 40 per cent of the completed mixture. The results of the experiments on carbohydrates are given in Table III.

From the results it appears that starch, sucrose, maltose, glucose, and fructose fall in one group in that they brought about little alteration in the extent of the intestinal reduction. These results may be explained, as already indicated, largely on the basis of rapid absorption of the sugars of this group. In the case of starch the reasons are not so clear but presumably any starch reaching

the large intestine is broken down too slowly for the sugars formed to have a pronounced effect on the intestinal flora. Lactose and dextrin belong in a class by themselves. They brought about a very marked diminution in the extent of intestinal reduction, lactose for example cutting it down from 45 to 18 per cent in one instance. The intestinal contents in these cases are acid in reaction and retain the red color due to ferric oxide instead of being blackened by reduction. There is also a laxative action and the

TABLE III.

Influence of Carbohydrates in the Diet on Intestinal Reduction.

Egg albumin diets. Albumin 20 per cent, agar 1 per cent, starch to make 100 per cent.

Carbohydrates.	20 per cent.	30 per cent.	75 per cent.
Starch.....	69		
Sucrose.....	72	82	69
Glucose.....	62	85	65
Fructose.....	77	79	
Maltose.....	72	87	72
Dextrin.....	40	55	42
Lactose.....	63	45	34

Meat-bread diets. Meat (dried) 20 per cent, bread 75 per cent, agar 2 per cent. Carbohydrates added to this basal diet.

Carbohydrates, 66 per cent.	Reduction.
	<i>per cent</i>
Starch.....	45
Sucrose.....	37
Lactose.....	18

feces are softer in character. The lessened reduction may be due in part to the fact that hydrogen sulfide is less rapidly reducing in acid than in neutral or alkaline solution, but whatever the causes involved it is apparent that lactose and dextrin definitely counteract the tendency toward the production of an intensely anaerobic condition in the intestine and in this way also cannot be without influence on the intestinal flora, inasmuch as the organisms of true putrefaction are obligate anaerobes. As already pointed out casein shows much less tendency to bring about reduc-

tion in the intestine than meat protein, egg albumin, etc., so that the peculiar efficacy of milk and modified milks in lowering reduction is readily explained on the basis of their composition, and the character of the flora to which they give rise.

The addition of 20 per cent of butter to a starch-albumin diet did not perceptibly alter the degree of reduction in the intestine.

Influence of Ordinary Foods.

A variety of common foods was next studied. The results of this series of experiments throw further light on the influence of different proteins and carbohydrates on the reduction of iron in

TABLE IV.

Influence of High Protein Foods on Intestinal Reduction.

Protein about 27 per cent, agar 2 per cent.

Food.	Reduction.
	<i>per cent</i>
Egg (boiled) 80, bread 75.....	56
Cheese 100, bread 75.....	38
Peanut butter 80, bread 15.....	28
Meat (cooked and dried) 20, bread 75.....	32
“ (raw) 67, bread 75.....	42
“ (“) 95.....	65
Milk (powdered) 95, agar 1.....	15
“ (“) 50, bread (dry) 45, agar 1.....	25
Soda crackers 95.....	44
Bread 95.....	50
“ (browned) 95.....	85

the intestines. In the first group of these experiments the amount of protein in the diets was kept fairly constant at about 27 per cent by the addition in some cases of dried bread as a supplement. 2 per cent of agar-agar was added to all diets and iron oxide made up 1 per cent in each case. The results are given in Table IV.

It will be seen that dried bread or soda crackers gave moderately high reductions (44 to 50 per cent). Bread toasted to a very dark brown was laxative and yet gave high reduction values. Milk has always been recommended as a food of value in the attempt to reduce intestinal putrefaction. Its recognized value and the findings for lactose and casein independently are amply confirmed

by the results of these experiments. Dried milk added to bread in the proportion 1:1 cut the reduction from 50 to 25 per cent and milk alone reduced this to 15 per cent which is a very low level for a food containing 27 per cent of protein. Milk was also added to bread-meat diets (see Table V) in different proportions. Making 50 per cent of the diet, reduction was cut from 32 to 16 per cent.

From the experiments on high protein foods it will be seen that raw meat when given alone led to a high reduction value (65),

TABLE V.

Influence of Fruits, Vegetables, and Milk on Intestinal Reduction.

Basal diet. Bread 75 per cent, meat (dry) 20 per cent, agar 2 per cent. Foods added to this diet.

Food.	Amount added.	Reduction.
		<i>per cent</i>
Basal diet alone.....		32
Milk powder.....	33	27
“ “	100	16
“ only.....		12
Bananas.....	100	31
Oranges.....	200	28
Apples (raw).....	200	23
“ (cooked).....	200	21
Raisins.....	100	32
Lettuce.....	100	31
Lettuce only.....		26
Cabbage (raw) only.....		22
Turnips (“) “		27

raw meat with bread a somewhat lower value (42), while very thoroughly roasted and dried meat gave with bread a still lower value (32). This would seem to indicate a higher putrescibility of raw meat in the intestine. Aside from any action of bacteria upon them, raw meats have in themselves a reducing action which has been made use of by placing them in the side arms of fermentation tubes to aid in the production of strictly anaerobic conditions. Inasmuch, however, as no reduction was noted in the small intestines of rats on raw meat diets and the reducing power of fresh meat must be considerably impaired by the action of the digestive

juices, the reducing properties of meat as such, and also of other fresh foods, in the intestine would seem to be less important than suggested by Herter (3).

Boiled eggs with bread gave higher values than cooked meat with bread, confirming the effect noted where powdered egg albumin was present in the diet. Cheese-bread diets gave lower values than eggs as would be expected from their content of casein. Peanut butter with very little bread gave low reduction figures. Certain vegetable proteins are generally believed to give rise to less putrefaction in the intestines than animal proteins of the meat and egg types. This would seem to be true of reduction also in the case of peanut butter, but seems less well supported by the figures obtained for breads and soda crackers. The higher temperature to which peanuts are subjected might not be without effect, and some labile sulfur might also be lost.

Fruits and certain vegetables are considered antiputrefactive because of their high carbohydrate:protein ratios and their laxative effects. Raw lettuce, cabbage, and turnips were given to rats as sole constituents of the diets. These gave low but no extremely low reductions (22 to 27 per cent): The following fruits and vegetable were tested out as additions to a standard bread and meat diet: oranges, apples (raw and cooked), raisins, and lettuce. These showed a tendency to give lower values than the basal diet, but the results were not so marked as might have been expected. The results of these experiments are given in Table V.

Influence of Intestinal Antiseptics, Etc.

Results of a few experiments on intestinal antiseptics are given in Table VI. No influence of salicylic acid could be noted. β -naphthol gave a decrease and then an increase. Kaolin has recently been suggested in the treatment of intestinal stasis as an adsorbent of toxins and a stimulant to peristalsis. Like naphthol, kaolin gave a decrease followed by an increase. Paper given *ad libitum* gave decreased reduction. Intestinal antiseptics have generally proven ineffective. Studies of the influence of some of these on the numbers of bacteria voided have shown a preliminary decrease as a consequence of increased peristalsis due to the irritant action of the drug followed by an increase presumably due to some degree of secondary intestinal stasis. The results

given here point in the same direction. Intestinal stasis was noted in several cases to be a factor of prime importance in increasing the reduction percentages and of more importance than dietary changes. The experiment on kaolin is an illustration of this. On the other hand, high degrees of reduction have been noted in rats with some intestinal disturbance resulting in diarrhea. Along several of the lines indicated further work is in progress, and will be required for a complete interpretation.

TABLE VI.
Influence of Antiseptics, Etc., on Intestinal Reduction.

Substance.	Contra- l diet reduction.	Reduction percentage on experimental period of days:		
		2	3	4
	<i>per cent</i>			
Salicylic acid, 1 per cent.....	71		67	69
β -naphthol, 0.5 per cent.....	34	17		47
Kaolin, 2 per cent.....	34	19	46	58
Paper <i>ad libitum</i>	27	2	7	12

CONCLUSIONS.

1. Studies were made of the comparative completeness of reduction of definite proportions of ferric oxide given with a variety of diets. The method of estimation given in a previous paper was found to be applicable to such studies on the albino rats which were used as subjects.

2. Reduction took place almost entirely in the cecum and large intestine and is, therefore, closely related to the processes of intestinal putrefaction of proteins which predominate in the lower bowel. Hydrogen sulfide is a substance concerned in this reduction process.

3. The influence of the character of protein in the diet was studied. Meat and egg proteins gave rise to high degrees of reduction in contrast with the low figures obtained for casein. Vegetable proteins were intermediate. Cooking of meat resulted in lessened reduction.

4. The carbohydrates, starch, sucrose, glucose, fructose, and maltose, had little effect in diminishing reduction. Dextrin and lactose produced the characteristic alteration of the intestinal

flora to an aciduric type and intestinal reduction was markedly diminished. Fat produced little effect.

5. A number of common foods were studied. Milk when given alone led to very low reduction values and when added to other diets decreased the reduction obtained. Milk thus occupies a position of special importance due to the peculiar protein and sugar it contains and their influence on the intestinal flora.

6. Fruits and green vegetables alone or as constituents of diets tended to give moderately low reduction values.

7. Antiseptics tested failed to produce any permanent decrease in reduction. Intestinal stasis led to marked increase in reduction.

8. Intestinal reduction as estimated by the method given is a useful index of intestinal putrefaction and gives additional information as to the degree of anaerobiosis that may exist in the intestine. As the organisms of true putrefaction presumably responsible for the more severe intestinal toxemias are anaerobic in character the information thus obtained should be of value.

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THE INFLUENCE OF SODIUM CHLORIDE UPON THE LEVEL OF BLOOD URIC ACID.*

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The present paper contains a description of some experiments on the effect of sodium chloride on the uric acid concentration in blood. It forms part of a larger piece of work in which we have been studying the effect of that salt upon the production of edema, with its consequent effect upon kidney function tests, during pregnancy. Its publication at the present juncture, however, is stimulated by the recent paper of Folin, Berglund, and Derick (1) upon the general problem of uric acid. Thus we find those authors making the statement that sodium chloride in doses of 30 gm. will cause an increased output of endogenous uric acid in the urine. This may be true, though the conditions under which sodium chloride will produce that effect are probably limited. The suggestion offered by those authors, that the increased output is due to a decreased concentration of uric acid in the blood, is probably correct also. Our own experiments are in support of such a conclusion. The present paper deals with the conditions and the mechanism whereby that result may be brought about. But until a similar mechanism is shown to be brought into play by such dissimilar substances as sodium chloride and amino acids we should hesitate to extend to all cases of increased endogenous uric acid output the same explanation. Arising from the same set of cir-

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cumstances we also find ourselves compelled to discuss a further generalization of those authors—that a high portein diet as contrasted with a low protein diet must inevitably coincide with a lower concentration of uric acid in the blood.

The subjects of these experiments were women in the latter half of pregnancy. All were considered as normal, with one or two exceptions which will be indicated in the course of the paper. Such disturbances as were present in any of them were of a slight nature only. The diets given are termed high protein and carbohydrate, the terms indicating an amount of the designated constituent in excess of that usually employed in a balanced diet. The diets were arranged to be at or slightly under the calory requirements of the subjects as calculated by the Du Bois standards. They were, however, never so far below the calory requirements as to necessitate a large utilization of the patient's own fat to make up the deficiency. (See diet list at end of Table II.) During the experimental periods the subjects remained in bed, and all urine collections, etc., were in charge of a special nurse. The bowels were regulated by an enema given daily. During the intervening periods the subjects were on hospital diet, and allowed to be around the ward. A certain amount of choice was allowed in diet during those times, but the same routine was proceeded with for bowel regulation and urine collections. In this matter we had the full cooperation of the patients. Blood specimens were always taken in the early morning before breakfast, using oxalate as anti-coagulant. Sodium chloride was determined in the urine by a modified method of Rappleye (2). Non-protein N in whole blood was determined by the technique of Folin-Wu (3); sodium chloride by the method of Whitehorn (4); uric acid by that of Benedict (5); and urea by the Van Slyke-Cullen (6) process. The serum proteins were determined refractometrically, using the conversion tables of Reiss (7).

In Table I are the results of a series of experiments showing the influence on the blood findings of the addition of 15 gm. of NaCl to a protein or carbohydrate diet. The subject was placed upon the diet for a period of 4 or 5 days, and received 3 gm. of NaCl. A blood specimen was taken, then the NaCl was increased to 15 gm. and the diet continued for a further 4 or 5 days. Table II further illustrates the method of experiment as it shows the details

of the urine collections and analyses in two typical cases. From motives of space the details of the other four cases are not given. They are, however, similar in character. The results show that the addition of a large amount of NaCl to a protein or carbohydrate diet at, or a little below, maintenance value is accompanied by a decrease in blood uric acid. The mechanism of this decrease would appear to be increased elimination of uric acid, for we find

TABLE I.

Effect of Addition of NaCl to a Carbohydrate or Protein Diet on the Level of Blood Uric Acid.

Date.	Subject.	Diet.	Per 100 cc. of blood.				Serum protein.	Average total urine N.
			Non-protein N.	Uric acid.	Urea N.	NaCl		
1924			mg.	mg.	mg.	mg.	per cent	gm.
Feb. 23	Le B.	Carbohydrate	28.0	4.00	12.9	456	7.20	4.94
" 28		+ NaCl.	22.0	2.94	11.2	477	6.55	4.10
" 23	M-rs-.	Carbohydrate	29.3	3.20	12.0	479	7.17	5.97
" 28		+ NaCl.	21.5	2.25	8.4	489	6.77	4.76
Mar. 11	Le B.	Protein	21.5	3.20	16.8	472	6.21	12.96
" 15		+ NaCl.	28.7	2.75	14.5	508	5.53	13.01
" 11	W-th-l.	Protein	30.0	4.25	21.9	470	7.67	14.35
" 15		+ NaCl.	38.7	3.88	18.7	495	7.52	16.89
" 31	Ri-dn.	Protein	36.0	4.20	14.0	436	6.27	11.18
Apr. 5		+ NaCl.	33.4	3.45	12.8	438	5.12	13.36
Mar. 31	W-w-d.*	Protein	39.5	4.34	12.0	441	6.34	15.30
Apr. 5		+ NaCl.	31.0	3.56	13.4	533	6.25	16.52

* Blood pressure = 150/96

a decrease in the percentage of serum proteins accompanied by a marked retention of NaCl and water, as judged by the urine analyses and the difference between the fluid intake and urine volume. The retention of water and NaCl takes place usually in the first 24 or 48 hours. It is sometimes accompanied by a faint edema. After 48 hours, however, an equilibrium is established in normal cases. The effect of the high NaCl intake is also

shown by the enhanced value of that constituent in the blood. The evidence which we have submitted, and which is true of all six cases, would point to a relative hydremia, or increased blood volume, being produced by the action of the NaCl, leading in turn to an increased elimination of uric acid through the kidney and hence a decreased concentration in the blood. We have not, unfortunately, determined the uric acid excretion in these cases. At the time we were more concerned with the clinical aspect of edema production in pregnancy and did not observe the effect upon

TABLE II.

Showing Retention of Water and NaCl on Addition of NaCl to Carbohydrate or Protein Diet.

Date.	Fluid intake.	Urine.		NaCl		Remarks.
		Volume.	Specific gravity.	Intake.	Output.	
Subject M-rs-. Calory requirements, 1,651. Diet 1,687 cals. High in carbohydrate.						
1924	cc.	cc.		gm.	gm.	
Feb. 19	1,105	1,600	1,014	3	5.50	Blood specimens taken
" 20	1,202	1,508	1,012	3	3.77	Feb. 23 and 28, 7.45
" 21	1,302	1,228	1,017	3	3.68	a.m., before break-
" 22	1,300	1,225		3	3.33	fast. See Table I for
" 23	1,240	840	1,024	15	7.64	analysis.
" 24	1,140	946	1,024	15	11.35	
" 25	1,280	902	1,022	15	10.95	
" 26	1,180	1,050	1,022	15	13.02	
" 27	1,400	1,452		15	10.77	
Subject Le B. Calory requirements, 1,534. Diet 1,595 cals. High in protein.						
Mar. 5	1,230	1,006	1,027	3	3.60	Blood specimens taken
" 6	1,030	1,018	1,028	3	3.65	Mar. 11 and 15, 7.45
" 7	1,030	1,016	1,025	3	4.06	a.m., before break-
" 8	1,230	972	1,026	3	3.88	fast. Very slight ede-
" 9	1,230	1,360	1,022	3	4.21	ma in legs on high
" 10	1,130	771		3	3.54	NaCl. See Table I for
" 11	1,670	820	1,034	15	7.45	analysis.
" 12	1,430	990	1,034	15	12.84	
" 13	1,510	1,018	1,034	15	15.34	
" 14	1,230	1,064	1,032	15	11.84	
" 15	1,670	1,513		15	16.47	

TABLE II—*Concluded.*

Carbohydrate diet.		Protein diet.	
	<i>gm.</i>		<i>gm.</i>
Oatmeal.....	100	Oatmeal.....	60
Sugar.....	40	Sugar.....	10
Milk.....	80	Skim milk.....	720
Bread.....	180	Fresh cod.....	85
Butter.....	25	Bread.....	200
Jam.....	80	Butter.....	23
Scraped beef.....	25	Scraped beef.....	230
Potato.....	130	Gelatin, diabetic.....	1 pkg.
Tapioca pudding.....	100	Potato.....	70
Boiled rice.....	110	Baked beans.....	110
Cream, 18 per cent.....	25	“ apple.....	60
Banana.....	80		
Orange.....	50		
Protein.....	30	Protein.....	118
Fat.....	34	Fat.....	38
Carbohydrate.....	304	Carbohydrate.....	185
Cals.....	1,687	Cals.....	1,595

TABLE III.

Comparison of Protein and Carbohydrate Diets on Level of Blood Uric Acid in Presence of Constant NaCl Intake.

Date.	Subject.	Diet.	Non-protein N.	Uric acid.	Urea N.	NaCl	Serum protein.
			<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
1924							
June 19	L-n-d.	Protein.	35.3	3.70	13.0	462	6.90
“ 23		Carbohydrate.	28.5	4.00	8.6		6.51
“ 19	S-v-g.	Protein.	33.8	3.33	11.6	445	7.24
“ 23		Carbohydrate.	31.7	3.28	8.6		7.08
“ 19	Le B.	Protein.	34.3	3.24	15.0	470	7.02
“ 23		Carbohydrate.	29.6	3.15	11.4		6.34
July 20	W-t.	Protein	24.4	2.03	6.9	462	6.92
“ 24		Carbohydrate.	30.2	2.44	9.1	462	7.02
Jan. 21	Le B.	“	21.0	3.11	10.0	519	6.86
“ 26		Protein.	46.8	3.15	14.5	517	6.63
“ 16	R-v-l.	Carbohydrate.	39.1	3.12		495	6.45
“ 21		Protein.	31.1	3.00	10.5	490	7.20

the uric acid until afterwards. We do not doubt, however, that the statement of Folin, Berglund, and Derick, that a high intake of NaCl leads to increased elimination of endogenous uric acid, holds true under our conditions. It may be noted in passing that our diets were not purine-free. They were, however, constant in composition for each subject, the same meals being given each day throughout the entire experimental period. The meals also were

TABLE IV.

Effect of Protein Diet on Uric Acid when Previous Diet Was Unknown.

Date.	Subject.	Diet.	Non-protein N.	Uric acid.	Urea N.	NaCl	Serum protein.
			<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		<i>per cent</i>
1924							
July 7	J-n-g.	Admittance.	30.0	4.61	8.85		
" 14		Protein.	34.9	3.55	41.6	503	7.06
" 15	W-t.	Admittance.	25.3	2.69	7.9	572	6.38
" 20		Protein.	24.4	2.03	6.9	462	6.92
Mar. 25	R-d-n.	Admittance.	23.6	2.68	7.9	474	5.90
" 31		Protein.	36.0	4.20	14.0	436	6.27
" 25	W-w-d.	Admittance.	22.5	2.75	7.95	457	6.77
" 31		Protein.	39.5	4.34	12.0	441	6.34
" 4	W-t-l.	Admittance.	39.5	3.75	14.5	485	7.59
" 11		Protein.	30.0	4.25	21.9	470	7.67
May 16	S-v-g.	Admittance.	28.5	2.78	10.9	446	7.26
" 20		Protein + NaCl.	29.6	3.00	9.8	438	7.61
" 23		" + "	27.3	3.03	12.58	453	6.98
" 16	T-l-n.	Admittance.	23.0	2.04	10.7	439	6.87
" 23		Protein + NaCl.	22.3	2.32	9.32	460	6.65

the same in food constituents for all subjects when on the same type of diet. The dietary conditions in this set of experiments were thus very constant.

The action of the NaCl is not confined to the lowering of the concentration of uric acid in the blood. In five out of six cases the concentration of urea is lowered. In four out of six cases the non-protein N is also lowered. It might be expected that the

non-protein N would vary in a manner similar to the urea, but as the non-protein N was carried out on whole blood, concentration of substances in the corpuscles other than urea would become a

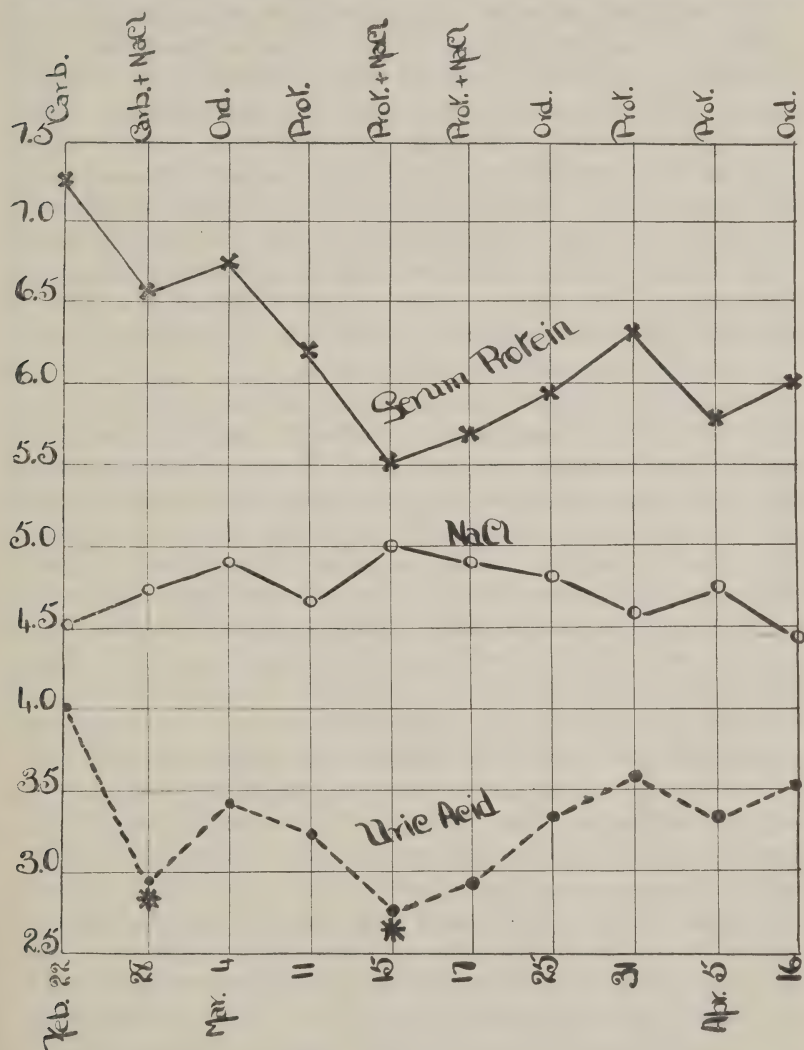


CHART 1. Record of Mrs. Le B. on carbohydrate and protein diets showing correspondence between curves of percentage of serum proteins and level of blood uric acid, and the inverse character of the curve of NaCl in blood.

factor, especially if such substances were not eliminated as rapidly as urea. Plass (8) has shown that such concentrations can take place.

As a result of this series of experiments we would ascribe the variations in the level of blood uric acid to the state of dilution or otherwise of the blood, provided we are dealing with normal healthy kidney tissue in order to allow of full elimination. The actual amount of uric acid to be eliminated would seem to be a minor matter provided the amount is not increased excessively. Such variations in amount as may be brought about by dietary alterations would seem to be dealt with rapidly and easily in spite of the kidney's lack of concentrating power for that substance as compared with urea or creatinine, for Denis (9) has shown that high purine diets have little or no influence on the level of blood uric acid, with normal kidney elimination.

That the level of blood uric acid is dependent mainly on the state of blood hydration is shown further in Chart 1. There are plotted from the record of subject Le B. the concentrations of uric acid, NaCl, and serum proteins over a considerable period of time and with varying changes of diet. The chart is of interest as it covers ten observations spread over a period of 53 days. In all instances the concentrations of serum protein and uric acid vary together in the same direction. This is in spite of the fact that the character of the diet varied greatly over this long period. The diet is indicated on the chart and it will be seen that it included the high carbohydrate and protein diets used in the experiments shown in Tables I and II, a further period of experimental high protein diet, and several periods in which the subject was allowed considerable choice of food. The periods thus include many variations in the amounts of exogenous uric acid. The periods of high salt feeding are marked by an asterisk, and it will be noted that they occur coincident with the lowest uric acid concentrations in the blood. Although we have emphasized the variations in the character of the food of this subject, yet the fundamental changes were never very great except at one point. It so happened that Le B. in exercising her choice of food preferred a high protein diet, so that diets marked as ordinary diets on the chart to contrast them with our experimental diets are really similar in fundamentals. The whole period then from March 4 to April 16 inclusive becomes

one of high protein diet. The one point at which the diet changed sharply in fundamentals was when on February 28 we changed from a carbohydrate to Le B.'s ordinary (or protein) diet. We have emphasized this somewhat at length because it is at this point that there occurs the only irregularity in the record of Le B. over this period. We refer to the concentration of NaCl in the blood on March 4. If the curves be examined, with the exception of this point, it will be noted that the concentrations of serum proteins and NaCl always run as complementary curves. We take it that under conditions of constant diet, such as we obtain in our series, the equilibrium is attained in the blood by the mutually corrective action of protein and Cl ion towards the action of water. If, however, a marked dietary change is made, such as that of a high carbohydrate to a high protein, thus involving a marked change in the amount of an excretory substance such as urea, then a new shift in ions may take place which may obscure the simpler relationships. We have, however, no desire to labor this point at present, as there are several objections which may be taken to our method of experimentation if we wish to push our conclusions beyond the simple statement, that a dilution of the blood is often balanced by an increase in its sodium chloride concentration, or that *vice versa* an increased concentration of sodium chloride in the blood brought about by increased intake is compensated by an increased blood volume; and that varying inversely with the state of hydration is the concentration of blood uric acid.

In their interesting and stimulating paper Folin, Berglund, and Derick note that the levels of blood uric acid are much lower in their subjects when on a high protein diet than when on a diet low in protein. Indeed when on a low protein diet the concentrations of blood uric acid in some of their subjects are surprisingly high, so much so that we should have hesitated to accept the figures as belonging to normal individuals had we ourselves not had evidence from another direction of the heights to which uric acid can rise in the blood under appropriate conditions. The decrease in the level of the blood uric acid brought about by the high protein diet is ascribed by Folin and his collaborators as due, in part at any rate, to an increased elimination through the kidney. From what we have written concerning the influence of sodium

chloride on the concentration of blood uric acid, it is evident that we should approach this question from the same standpoint of blood hydration. Any experiments designed to test this point must necessarily be under conditions of constant NaCl intake. Table III contains the results of six such experiments. The periods of high protein or high carbohydrate food were of from 3 to 5 days, with a constant NaCl intake of 3 gm. The diets were also isocaloric for each individual. The effect upon the uric acid concentration in the blood is indeterminate. It is lower in three cases when under the influence of the high protein, but the changes in any case are small. Nor was there any evidence of retention of water or NaCl to indicate a probable hydremia when on either diet. The serum proteins are higher in four cases when on the high protein diet, rather than lower as we might expect from a consideration of Folin's experiments and our own arguments. Changes in the percentage of serum proteins, however, observed without corresponding water changes, cannot be taken as an indication of the state of hydration of the blood, especially when the albumin and globulin are measured together by means of the refractometer. In order to obtain further evidence we have examined our records to see what has been the effect on the blood uric acid when we have placed our subjects on a high protein diet immediately on their entrance to the ward, thus offering a contrast with their ordinary previous diet. The results are collected in Table IV. In the first five cases the diets were of maintenance calory value, but as can be judged by the urea N concentration in the blood they were much higher in protein content than the diet previous to admission. W-t. may be a possible exception. In the last two cases the diets were not only high in protein, but contained an excess of calories, and 15 gm. of NaCl in addition. Of the first five cases the uric acid is lowered in two and increased in three. The two cases showing lowered blood uric acid figures showed also from the urine analyses large retentions of water. The other three cases showing a rise in uric acid, although two showed an apparent water retention, showed at the same time a loss of NaCl. The diet given these cases contained only 3 gm. of NaCl, and it is probable that this low amount of salt represented an intake less than that to which they were accustomed, and hence led to a diminished blood volume. Such an explanation though it is open

to criticism nevertheless accords with the probabilities of the case. In the last two cases of Table IV where an excess number of calories were given, certainly the amounts of both protein and NaCl were far greater than those in their ordinary dietary. *A priori*, it would be expected that the uric acid in the blood would be found at a lower level than at their admittance, yet in both cases the uric acid showed a slight rise. Of the two T-l-n. showed a marked retention of water and NaCl. The serum proteins are lowered and the NaCl concentration in the blood is increased. We can only conclude that the excess calories of the diet interfered in some way with the elimination of uric acid. Case S-v-g. is equally difficult of interpretation. Here the full amount of NaCl was excreted in the urine on the 1st day without any sign of water retention. The serum proteins first rise and then fall. The NaCl concentration of the blood moves in the opposite direction; yet the uric acid shows a steady rise. Again we can only conclude that excess calories have been the cause. Whatever may be the explanation of those results the whole trend of argument from the figures shown in Table IV is to drive us to the conclusion that a high protein diet in itself does not necessarily act as a blood uric acid depressant. Yet Folin, Berglund, and Derick show three cases (H-w-d., W-n., B-ck.) where a high protein diet apparently induces a lowering of the blood uric acid. The average figures of their subjects are certainly in favor of such a conclusion. It is true that our own high protein diets were not purine-free. They would not, however, be described as purine-rich diets. Subject H-w-d. of Folin, Berglund, and Derick on a purine-rich, high protein diet showed a plasma uric acid of 5.4 mg. per 100 cc. It is true this is greater than when on a purine-free, high protein diet (3.4 mg.), but it is less than one of the figures of the same subject when on a purine-free, low protein diet (6.0 mg.). Moreover, our figures for the concentration of blood uric acid on our high protein diets are not uniformly higher than on our high carbohydrate diets, as they would be were the purine content of the former responsible for any increases which we have observed. The cause of the discrepancy between the results of Folin, Berglund, and Derick and ourselves as to the effect of a high protein diet upon the level of blood uric acid must be sought in other directions. And in this connection we feel that it is pertinent to enquire why the levels of

the blood uric acids of the subjects of these investigators are so uniformly high when on purine-free, low proteins diets. Figures of 5.0, 5.4, 5.6, and 6.0 mg. of uric acid per 100 cc. of plasma are not within the usually accepted range of normal variation of that substance, yet these are the figures shown by four of the subjects of Folin and his collaborators. Our subjects on low protein diets (high carbohydrate) show no such high values. Our figures are determined on whole blood while those of Folin and his collaborators are on plasma. The highest blood uric acid we have observed on a carbohydrate diet has been 4.0 mg. per 100 cc. in the case of Mrs. Le B. on February 22 (see chart). This would correspond to 4.66 mg. per 100 cc. of plasma calculated on a 50 per cent corpuscular volume and a 2:1 distribution ratio of uric acid between corpuscle and plasma. We have also observed figures of 5.26 and 4.87 mg. of uric acid per 100 cc. of plasma in normal men. These high normal figures for uric acid in plasma are associated with high concentrations of serum proteins. Thus the percentage of serum proteins ran 7.2, 9.1, and 8.1, respectively, in the three cases. The two latter are undoubtedly high and the first figure is the highest observed in that individual for many subsequent weeks.

We cannot conclude without drawing attention to the parallel between the action of sodium chloride under the conditions we have defined, and that of the pharmacological blood uric acid depressants, sodium salicylate, cincophen (atophan), and neocincophen (novatophan). These drugs are known to increase the elimination of uric acid (10), and to depress the level of blood uric acid (11). Their action in this direction is known to be rapid and limited, and once having brought about diminution in the blood uric acid increased dosage is not followed by increased action. Their action is not confined to uric acid but extends, though not perhaps so markedly, to urea and creatinine (11). They have been shown to act as antipyretics by increasing blood volume in fever, though this action has not been capable of demonstration in normal individuals (12). Sodium chloride is stated by Folin, Berglund, and Derick to increase the output of endogenous uric acid. We have shown that it acts as a blood uric acid depressant, doing so by virtue of its ability to increase blood volume under however limited conditions of diet. Its action is rapid, but once the new

equilibrium is established further action is impossible, though we do not know whether 15 gm. is the maximal effective dose. It shows a similar effect upon blood urea and non-protein N. The parallel is not complete; there are points of dissimilarity, but it is sufficiently striking to make it worth while to draw attention to it.

SUMMARY.

The effect of 15 gm. of NaCl added to a protein or carbohydrate diet is to lower the level of blood uric acid.

This is accompanied by an increase of NaCl in the blood, a decrease in serum proteins, and a total retention of water and NaCl.

The decreased concentration of uric acid in the blood is believed to be brought about by an increased hydration of the blood, resulting in increased elimination of uric acid.

On a constant NaCl intake little difference is found in the level of the blood uric acid when on protein or carbohydrate diet.

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AMINO ACID SYNTHESIS IN THE ANIMAL ORGANISM.

THE AVAILABILITY OF SOME CAPROIC ACID DERIVATIVES FOR THE SYNTHESIS OF LYSINE.*

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In the functioning of body cells, amino acids, which are carried to the cells by the blood stream and lymph after absorption from the alimentary canal or which are stored in the cell protoplasm as protein, are constantly catabolized. This process varies in its intensity; the amino acid may be completely broken down to water, carbon dioxide, and ammonia, apparently furnishing energy only, or a partial degradation may result in a conversion of fragments of the amino acids into products having a vital function in the various processes of the body. Thus, the tyrosine liberated in the cells presumably gives rise to adrenalin; tryptophane is believed to be necessary for the formation of thyroxin, the active principle of the thyroid; and cystine is probably essential for the synthesis of glutathione which is of such vital importance in oxidative processes in the body. If these amino acids are not furnished to the cell they must be synthesized from other metabolites present and if this is not possible, malnutrition results, growth ceases, reproduction is interfered with, and death may follow.

If the growing animal be furnished, not with the amino acid itself, but with certain closely related derivatives of that amino acid, the other nutritional requirements being satisfactory, can it synthesize the necessary unit from the derivatives or can it

* An abstract of a thesis submitted by Daniel A. McGinty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Michigan.

utilize the derivatives themselves for growth and development? Such a study is presented in the present attempt to determine whether the organism of the young white rat may utilize certain amino and hydroxy derivatives of caproic acid closely related to lysine (α , ϵ -diaminocaproic acid) as precursors of lysine for growth.

The validity of the experimental method used in these studies is conditioned by the indispensability of lysine for normal growth of the young white rat. The most decisive evidence of the rôle of lysine in nutrition is that of Osborne and Mendel (1, 2) in which they demonstrate that gliadin, the alcohol-soluble protein of wheat gluten, as the only source of protein in the diet of young rats, did not permit growth. The animals, however, maintained their body weight at quite constant levels. The failure of growth was presumably due to the low lysine content (1.21 per cent) of gliadin (3), although this lysine content was apparently sufficient for maintenance. Gliadin, when supplemented with lysine, allowed practically normal growth, thus proving that gain in weight is dependent on the presence of lysine, and that lysine is a limiting factor in the amino acid content of gliadin. Experiments with zein (1), lacking in tryptophane and lysine, as the source of protein, have also demonstrated the necessity of lysine for growth. Hart, Nelson, and Pitz (4) have proven that the mammary gland of the lactating female rat is unable to synthesize lysine. Buckner, Nollau, and Kastle (5) fed chicks on grains high in lysine. Those on the lysine-deficient grains showed decidedly slower growth and development. Osborne and Mendel (6) obtained results agreeing with those of Buckner, Nollau, and Kastle.

Abderhalden (7, 8) demonstrated that the use of hydrolyzed casein, from which lysine had been removed, as a source of protein for adult dogs led to a negative nitrogen balance which was slightly improved if lysine was returned to the diet. Abderhalden (9) also found that body weight of an adult rat fell rapidly if lysine was lacking from the synthetic diets, but that this loss in weight was regained when lysine was again incorporated into the food. Other investigators confirm the views of the indispensability of lysine, although Geiling (10) does not agree with the opinion of Hart, Nelson, and Pitz (4) that lysine is necessary

for adult maintenance. McCollum, Simmonds, and Pitz (11) agree that lysine is necessary for growth, although they do not state that it is indispensable for adult maintenance.

The possibility of lysine synthesis from simpler caproic acid derivatives has not been extensively studied. Embden and Schmitz (12) have demonstrated by perfusion experiments with the surviving liver that α -hydroxy and α -keto acids may be converted to the corresponding amino acids. That this reaction which can readily be demonstrated in perfusion experiments also occurs in the intact living organism has not been proven, although some evidence in support of this may possibly be found in the experiments of Grafe and Schläpfer (13) and Abderhalden (14).

Of the various hydroxy and amino derivatives of caproic acid of interest in connection with the problem of lysine synthesis, the following were available for the present study, α -hydroxy-, ϵ -hydroxy-, ϵ -amino-, and α -hydroxy- ϵ -aminocaproic acids. In addition, nor-leucine (α -aminocaproic acid) has already been shown by one of us (15) to be unavailable as a lysine precursor. Of these derivatives the synthesis of lysine from α -hydroxy- ϵ -aminocaproic acid seemed most probable in the light of other studies. The only chemical change necessary to form lysine from α -hydroxy- ϵ -aminocaproic acid would be a reversal of the hydrolytic deamination process which is thought by some to be possible in the living organism (12, 13). Likewise, α -amino- ϵ -hydroxycaproic acid might be considered to be easily converted into lysine by a reversal of the deamination process on the ϵ carbon atom. If both the α and ϵ carbon atoms are capable of amination by union of the hydroxy groups with ammonia, then α , ϵ -dihydroxycaproic acid should likewise be capable of lysine synthesis. Considering those derivatives in which amination would have to take place by replacement of a hydrogen atom, possibility of synthesis is less likely. This is especially true in view of the work of Lewis and Root (15) who attempted to replace lysine by nor-leucine in the food of growing rats. They fed diets in which gliadin formed the protein fraction. In the case of nor-leucine, the α -amino group is already present in the molecule, and since there is some evidence that the ϵ -amino group of lysine does not function in the peptide linkage, it was

thought that the animal organism might utilize nor-leucine without further substitution of amino groups in the formation of new protein available for growth. They found, however, that neither the inactive *dl* form nor the active *d*-nor-leucine was able to supplement gliadin as lysine does, indicating that there was neither a synthesis of lysine from nor-leucine, nor a utilization of nor-leucine in place of lysine. Since nor-leucine is not to be considered as a precursor of lysine, one must probably exclude ϵ -aminocaproic acid, α -hydroxycaproic acid, and ϵ -hydroxycaproic acid on the same grounds, that amination of a carbon atom, without the hydroxyl group as an intermediate stage, is apparently not possible.

EXPERIMENTAL.

With one exception (Rat 69, Chart IV), the experimental animals used were young white rats which were placed on the experimental diets at an age of about 5 weeks when the weight was from 45 to 65 gm. In general, the laboratory procedure described by Miss Ferry (16) of the Osborne and Mendel laboratory was employed. The rats were bred in the laboratory. No disease occurred in the colony during the course of the experiments. The casein used as a food for control animals was a commercial product (Lister Bros.' white casein), which was extracted twice with hot alcohol for 30 minutes to remove lipoids partially. Gliadin was prepared in the laboratory. The inorganic salt mixture used was that of Osborne and Mendel (17). The corn-starch and lard were commercial products, the same brands being used throughout. The water-soluble vitamin was furnished by a yeast vitamin powder (Harris Laboratories), approximately 50 mg. of this powder being fed daily in the form of a pill made with equal parts of vitamin powder and starch. This vitamin preparation contained no protein and supplied little nitrogen. An analysis of this powder made by Miss M. L. Long of this laboratory showed the following composition: nitrogen, 8.11 per cent; sulfur, 0.71 per cent; phosphorus, 5.43 per cent; ash, 24.11 per cent; moisture, 7.04 per cent; amino nitrogen (Folin) before hydrolysis, 1.66 per cent, after hydrolysis, 2.37 per cent; cystine (Folin and Looney) before hydrolysis, 1.18 per cent, after hydrolysis, 1.46 per cent. The fat-soluble vitamin was supplied by cod liver oil.

Lysine was prepared in the laboratory by acid hydrolysis of casein by the method of Kossel and Kutscher described by Weiss (18). This method was modified in that the first neutralization was made with calcium hydroxide instead of barium hydroxide. The lysine picrate of Kossel and Kutscher was converted into the hydrochloride by extraction of a dilute hydrochloric acid solution of the picrate with ether to remove the picric acid. The extracted liquid was then evaporated on a water bath to dryness, dissolved in methyl alcohol, and precipitated as lysine dichloride

TABLE I.

Composition of Diets and Values of Caproic Acid Derivatives in Terms of Gliadin.

	18 per cent protein.	15 per cent protein.	12 per cent protein.	9 per cent protein.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Protein.....	18.0	15.0	12.0	9.0
Salt mixture.....	4.5	4.5	4.5	4.5
Sucrose.....		3.0	4.0	4.5
Starch.....	50.0	50.0	52.0	54.5
Lard.....	24.5	24.5	24.5	24.5
Cod liver oil.....	3.0	3.0	3.0	3.0
Total.....	100.0	100.0	100.0	100.0

Equivalent nitrogen values of the caproic acid derivatives added to gliadin diets.

1 gm. of lysine (1.486 gm. of lysine dichloride) replaced 1.2 gm. of gliadin.

1 " " ϵ -aminocaproic acid replaced 0.66 gm. of gliadin.

1 " " α -hydroxy- ϵ -aminocaproic acid replaced 0.60 gm. of gliadin.

1 " " cystine replaced 0.66 gm. of gliadin.

by absolute ethyl alcohol. Lysine was fed as the dihydrochloride. In certain experiments, synthetic *dl*-lysine was used to supplement gliadin. The caproic acid derivatives studied and the synthetic *dl*-lysine were prepared under the direction of one of us (M.) in the Laboratory of Organic Chemistry of the University of Illinois. An account of their preparation and properties will be published elsewhere.¹

The food mixtures were made up frequently so as to insure freshness and kept in an ice box. In those experiments in which lysine or caproic acid derivatives were incorporated in the diet along with the gliadin, a portion of the gliadin, equivalent in

¹Marvel, C. S., *J. Am. Chem. Soc.*, 1924, xl (in press).

its amount of nitrogen to the nitrogen of the lysine or caproic acid derivatives added, was removed. In this way the total nitrogen content of the diet remained the same. In Table I is presented the composition of the various diets with the equivalents in terms of gliadin of lysine and the caproic acid derivatives fed.

Rats were always given gliadin diets previous to addition of caproic acid derivatives in order to demonstrate in each rat the failure of growth on such a diet. Control experiments with casein were made on each litter of rats to demonstrate the possibility of normal growth with this adequate protein. In all cases where caproic acid derivatives were used, lysine subsequently replaced the derivative in question to demonstrate the adequacy of a gliadin plus lysine food for each individual animal.

DISCUSSION.

The results of the experiments are shown in Charts I to VI. In all cases, the food intake was carefully measured, but in order to condense the data, it is not recorded for the individual animals in this paper. The rate of growth of the control rats on casein food was uniform and for the most part was practically the same as that of stock rats on milk, bread, and cabbage diets. Chart I shows the failure of any considerable growth on a gliadin diet. This is not due to a smaller food intake since the data indicated that there was an even greater food consumption per 100 gm. of rat on gliadin diets than on casein diets. That the failure of normal growth on gliadin diets continues for long periods is shown by Rats 37 and 48 (Chart I) which were studied for periods beyond those shown on the chart. These rats were kept on a lower level of protein intake and it is apparent that even with 15 per cent of gliadin, there is perfect maintenance and even a slight gain in weight. This question of protein level will be referred to later.

The curve of growth of Rat 15 (Chart I) shows very strikingly the effect of adding 0.5 per cent of lysine to the gliadin. This is not the result of an increased food intake, there being, in fact, a slightly decreased consumption of food. In this case, the lysine is equal to practically 2.8 per cent of the protein of the food mixture. The growth curves of other animals in subsequent

charts also illustrate clearly the growth-promoting effect of the addition of small amounts of lysine to gliadin diets. Attention should be called to the slow rate of growth of Rat 6 (Chart V, Diet K). The gliadin in this period was supplemented with impure lysine whose potency we wished to determine. This same sample of lysine was given to Rat 5 (Chart V, Diet K) with more satisfactory results. The results confirm the earlier work of Osborne and Mendel in which protein-free milk was used as the source of carbohydrate and inorganic salts and which has been questioned because of the possible occurrence of small amounts of nitrogenous compounds in the protein-free milk.² In the present series of experiments the only significant source of extra nitrogen is found in the nitrogen of the yeast vitamin powder which, in the amounts fed (50 mg. daily), would approximate 4 to 5 mg. of nitrogen daily.

The failure of α -hydroxycaproic acid to supplement gliadin as does lysine is indicated in Chart III. The rates of growth of rats on diets of gliadin supplemented with α -hydroxycaproic acid were not greatly different from those of rats on gliadin alone, whereas, when the same rats were given a gliadin plus 0.5 per cent lysine food, their gains in weight were quite pronounced. Rat 7 during 35 days on an 18 per cent gliadin diet made no gain in weight; during 42 days on a diet of 18 per cent gliadin plus 1 per cent α -hydroxycaproic acid there was a gain of only 7.5 gm.; on a 18 per cent gliadin diet plus 0.5 per cent lysine diet, there occurred in 32 days a gain of 58.5 gm. It may be argued that the rats ate less food while on the gliadin and gliadin plus α -hydroxycaproic acid diets than on gliadin plus lysine diets and that their failure to grow was due to a diminished food intake. However, in two cases, Rats 7 and 44, there was actually less food eaten per unit of body weight on a gliadin plus lysine diet than on gliadin plus α -hydroxycaproic acid diets, and in the

² Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1924, lix, 339) have recently discussed the supplementary value of "protein-free milk" and conclude that "obviously, therefore, no great part of the nitrogen of the protein-free milk belongs to any one of the essential amino acids; and, consequently, it cannot supplement to an important degree any amino acid deficiency of the proteins of the diet unless the latter are fed in very small proportion."

other rats of this series, while there was a slight increase in the amount of food consumed on the lysine diets, the increase in weight was entirely out of proportion to the increased food consumption. For example, Rat 13 (Chart III) during 42 days on a diet of 18 per cent gliadin plus 1 per cent α -hydroxycaproic acid showed a gain of 5.5 gm. with an average weekly food intake of 35.4 gm. In the next period of only 35 days, on a diet of 18 per cent gliadin and 0.5 per cent lysine, the food consumption was 5 gm. a week greater, but the gain in weight per 100 gm. of food was over six times that of the preceding period.

In view of the fact that gliadin contains a certain small percentage of lysine it was considered possible that rats fed the 18 per cent gliadin diets might not experience an acute shortage of lysine and that rats maintained on lower levels of gliadin where the deficiency in lysine was even greater than on an 18 per cent level, might develop a greater synthetic power of converting the caproic acid derivatives into lysine. For this reason, it was considered necessary to determine the minimum amount of gliadin, which, when supplemented with lysine, would allow rates of growth comparable to those on 18 per cent casein. Chart II summarizes the results. Rats 19 and 20 on 9 per cent gliadin diets show a comparatively rapid decline, especially in the case of Rat 19, although the food intake in both cases was satisfactory. When a 9 per cent gliadin food was supplemented with 1 per cent lysine, the rate of growth was greatly below that of a rat on 18 per cent gliadin plus 0.5 per cent lysine and only slightly greater than that of rats on 18 per cent gliadin alone. For example, Rat 21, on Diet B (Chart II), which consists of 9 per cent gliadin plus 1 per cent lysine, showed a gain of only 6 gm. in 35 days, only slightly greater than that of Rat 15 (Chart I) which made a gain in the first 35 days of 5.5 gm. on an 18 per cent gliadin diet. This same animal (Rat 15), on a diet of 18 per cent gliadin supplemented with 0.5 per cent lysine, gained 52.5 gm. in 35 days. It is evident that on a gliadin intake level of 9 per cent, other dietary inadequacies than lack of lysine become apparent, either an insufficient intake of total nitrogen or a deficiency in some other amino acid.

Rat 20, on a 12 per cent gliadin diet, made a slight increase in weight, 2 gm. in 42 days. However, when 12 per cent gliadin

was supplemented with 1 per cent lysine, the gain did not approximate that of control animals on a casein diet. This is further illustrated in the curves of growth for Rats 19, 22, 23, and 24. It was thought that failure of growth on these low gliadin levels might have also been due to a lack of cystine, or that an extra supply of cystine, because of its supposed relationship to oxidative function, might accelerate body metabolism and cause a more efficient utilization of gliadin. Rat 21 (Chart II, Diet C) shows that added cystine does not change the slope of the curve from that obtained with Diet B which contains 9 per cent gliadin with 1 per cent lysine. On a diet of 9 per cent gliadin plus 1 per cent cystine, there was practically no growth. It was found that a gliadin level of 15 per cent was adequate for maintenance and that this was the lowest level of gliadin which, when supplemented with 0.5 per cent lysine, would allow rapid growth. Subsequent to this phase in the work, all diets were prepared with 15 per cent gliadin.

There was available a limited amount of the sodium salt of ϵ -hydroxycaproic acid so that it was possible to conduct experiments on one rat (hooded rat) only. The result is shown on Chart IV, Rat 69. ϵ -hydroxy-, like α -hydroxycaproic acid, failed to influence the rate of growth.

When ϵ -aminocaproic acid was supplied with the gliadin, the gain in weight of rats was approximately the same as the gains on diets of gliadin alone (Chart V). When cystine was added to a diet of 18 per cent gliadin plus 1 per cent ϵ -aminocaproic acid (Rat 8, Diet O) it had no effect of causing a better utilization of this caproic acid derivative. However, substitution of lysine itself for the lysine derivative resulted in prompt growth. ϵ -Aminocaproic acid in these experiments, and α -aminocaproic acid, as previously shown (15), are evidently not available as precursors for lysine.

As with α -hydroxy-, ϵ -hydroxy-, and ϵ -aminocaproic acids, the results with α -hydroxy- ϵ -aminocaproic acid were negative (Chart VI). The addition of α -hydroxy- ϵ -aminocaproic acid failed to influence the rate of growth in any way, while, in the same rats, the response to the addition of lysine was satisfactory. The addition of cystine had no stimulatory effect, nor did it in any way effect the food intake (Rat 10, Chart VI).

The inability of the growing rat, under the conditions of these experiments, to synthesize lysine from the possible precursors studied, is not in accord with the perfusion experiments of Embden and Schmitz (12) nor does it lend support to the theory of protein synthesis from non-nitrogen rests and ammonium salts or urea as suggested by the feeding experiments of Abderhalden (14) and Grafe and Schläpfer (13). Theoretically, the results are disappointing. According to Neubauer (19) and others the first step in amino acid catabolism is conversion to the corresponding keto acid or keto aldehyde, and according to Kotake (20), under conditions of hydrolytic deamination, the corresponding hydroxy acid may also be formed. One would expect, under conditions in which there was a great demand for lysine as with the gliadin diets that, if the reversal of the hydrolytic deamination were at all possible, it should occur in this case. Equilibrium reactions, theoretically, are reversible, even in the living cell.

With α -hydroxy-, ϵ -hydroxy-, and ϵ -aminocaproic acids, a more complicated process of synthesis would be necessary to manufacture lysine and with these derivatives synthesis might not have been expected, especially in view of the fact that nor-leucine had been shown to be unavailable as a lysine substitute. However, with α -hydroxy- ϵ -aminocaproic acid, synthesis should be quite readily accomplished; the ϵ -amino group was already present and the amination of a hydroxy group has been shown to occur, at least in perfusion experiments. One may argue that the enzyme that caused a hydrolytic deamination could not reverse the process because of its intrinsic specificity, but there is existing evidence that certain lipases are able to reverse the process of saponification. Certain enzymes among the carbohydrate-splitting group are likewise capable of carrying out reversible reactions.

A possible explanation is that ammonia is not available in the same cell or in the same part of the cell in which construction of new tissue takes place. It is also possible that the conversion of ammonia into urea in the cells is so rapid that ammonia for amination of hydroxy groups is not available. This, however, hardly seems probable in view of the increased ammonia formation in response to acidosis. A non-absorption of the caproic acid derivatives might explain their failure to replace lysine in the

food. This is not likely in view of the close chemical relationship to lysine which is absorbed and utilized. Furthermore, Greenwald (21) proved that nor-leucine was absorbed and in normal animals yielded urea. In the phlorhizinized dog, its carbon was converted into glucose.

In the experiments described the α -hydroxy- ϵ -aminocaproic acid fed was optically inactive, the *dl* form. It was considered that the failure of lysine synthesis with this compound might have been due to the inability of the organism to utilize the *dl* form for synthesis. This explanation would be invalid if it could be shown that the organism of the rat could utilize *dl*-lysine as a supplement to gliadin instead of the naturally occurring *d*-lysine. The results of experiments with *dl*-lysine as a supplement to gliadin are shown in Charts III (Rat 17) and IV. The curves show a good utilization of the synthetic *dl*-lysine at levels of 1 per cent (equivalent to 0.5 per cent *d*-lysine). In the one experiment (Rat 68, Chart IV) in which 0.5 per cent of the *dl* compound was fed, growth did not occur as rapidly as with 0.5 per cent *d*-lysine. In the experiment with Rat 67 the food intake was greatly diminished during the period of the addition of the natural lysine to the diet, so that the failure of growth on this diet is readily explained.

The evidence presented seems to indicate that under the experimental conditions, lysine cannot be synthesized from closely related precursors. Furthermore, the evidence is not in support of the theory of a union between ammonia and the hydroxy groups of α -hydroxy fatty acids to form α -amino acids at least in the living organism. This is not in accord with the views of Embden and Schmitz (12) who perfused surviving organs, or of Abderhalden (14) and Grafe and Schläpfer (13) who studied the nitrogen balance after the administration of ammonium salts.

We believe, as the result of a careful consideration of the literature, that there is available no entirely satisfactory evidence of amino acid synthesis from ammonia and non-nitrogenous rests in the organism of the *living animal*.

SUMMARY.

1. The value of four caproic acid derivatives, α -hydroxycaproic, ϵ -hydroxycaproic, ϵ -aminocaproic, and α -hydroxy- ϵ -

aminocaproic acids, as supplements to the incomplete protein gliadin, has been studied in the white rat. None of these caproic acid derivatives could supply the deficiency of the protein (gliadin) element of the diet and promote growth as did lysine. They may not, therefore, be considered as available precursors of lysine.

2. Since growth was possible on diets of gliadin supplemented with *dl*-lysine, the failure of α -hydroxy- ϵ -aminocaproic acid to replace lysine for growth cannot be explained on the basis of the inability of the organism to utilize the *dl* form in body reactions.

3. The evidence presented indicates that under the experimental conditions of the present study, α -hydroxy acids are not capable of conversion by the animal organism into α -amino acids. This is not in accord with theories of conjugation of ammonia with a non-nitrogenous rest to form amino acids.

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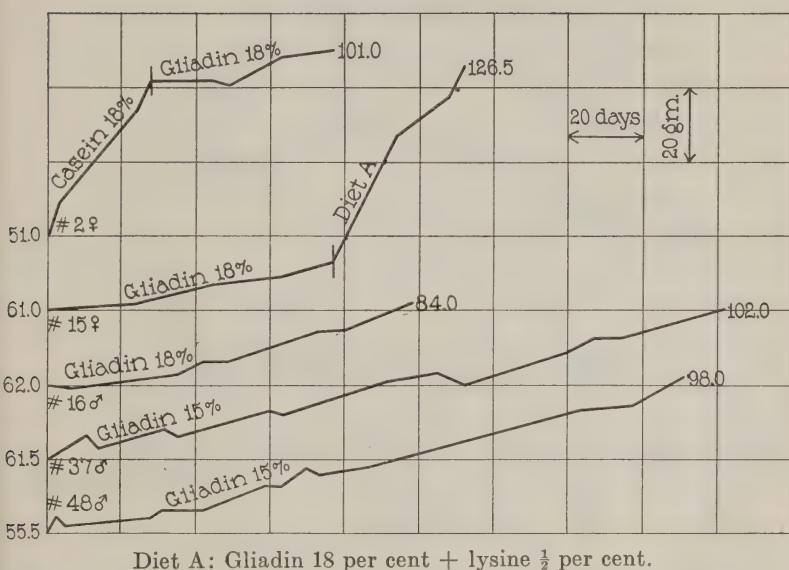
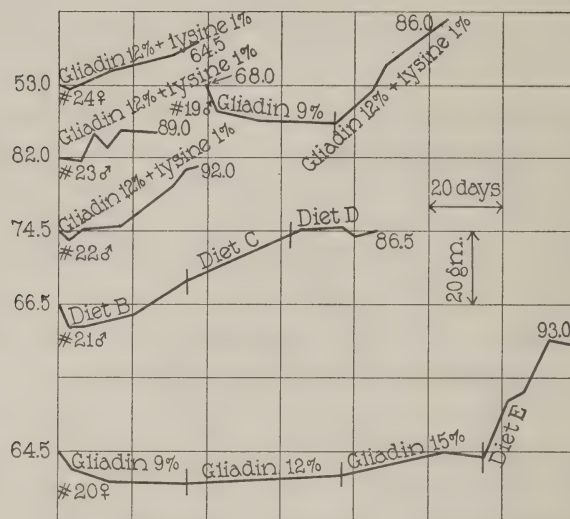


CHART I. Showing the curves of growth of rats on diets containing gliadin as the source of protein. It will be seen that there was some gain in weight, though the gains were not comparable to those with casein diets. The prompt response in growth when 0.5 per cent of natural lysine supplemented the gliadin is to be noted in the curve for Rat 15. With Rat 2, prompt cessation of growth in a fair sized rat was observed when casein was replaced by gliadin. The experiments with Rats 37 and 48 were continued beyond the period shown in the chart. At the end of 44 and 42 weeks, respectively, they weighed 111.0 gm. (Rat 37) and 118.0 gm. (Rat 48).



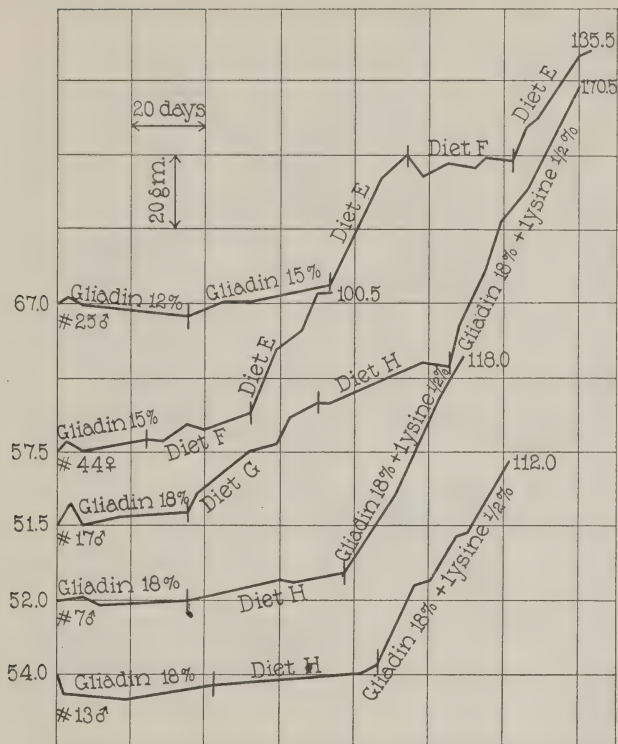
Diet B: Gliadin 9 per cent + lysine 1 per cent.

" C: " 9 " " + " 1 " " + cystine 1 per cent.

" D: " 9 " " + cystine 1 " "

" E: " 15 " " + lysine $\frac{1}{2}$ " "

CHART II. Showing the effect of low levels of intake of gliadin. Curves 19 and 20 show the inadequacy of gliadin for maintenance when fed at a level of 9 per cent of the total food. The replacement of this low protein intake by a higher level of 12 per cent gliadin (Curve 20) does insure maintenance, but inspection of Curves 22, 23, and 24 will show that when 12 per cent gliadin is supplemented with 1.0 per cent lysine normal growth fails to result. 9 per cent gliadin supplemented with 1.0 per cent lysine did not permit normal gain in weight (Curve 21, Diet B). The curve for Rat 21 (Diet C) shows that the addition of cystine to a 9 per cent gliadin + 1.0 per cent lysine food does not increase the rate of growth and demonstrates the prompt cessation of growth (Diet D) when the lysine was removed. Curve 20 shows that a 15 per cent intake of gliadin was quite sufficient for maintenance and when supplemented with lysine (Diet E) allows rapid growth.



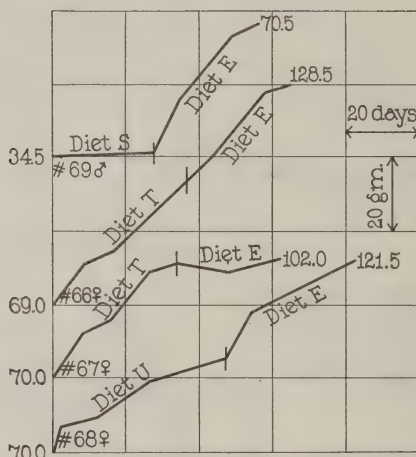
Diet E: Gliadin 15 per cent + lysine $\frac{1}{2}$ per cent.

" F: " 15 " " + α -hydroxycaproic acid 1 per cent.

" G: " 18 " " + synthetic *dl*-lysine 1 " "

" H: " 18 " " + α -hydroxycaproic acid 1 " "

CHART III. Showing the failure of α -hydroxycaproic acid to satisfactorily supplement gliadin on levels of 15 and 18 per cent protein intake. If either diet was supplemented with lysine, normal growth followed. Curve 17 (Diet G) shows the effect of supplementing 18 per cent gliadin with synthetic *dl*-lysine. Apparently, only the natural optical isomer is utilized completely. 1 gm. of synthetic *dl*-lysine dichloride was used, which is equivalent to 0.66 per cent of *dl*-lysine and only 0.33 per cent of the natural isomer, *d*-lysine. Compare also Chart IV.



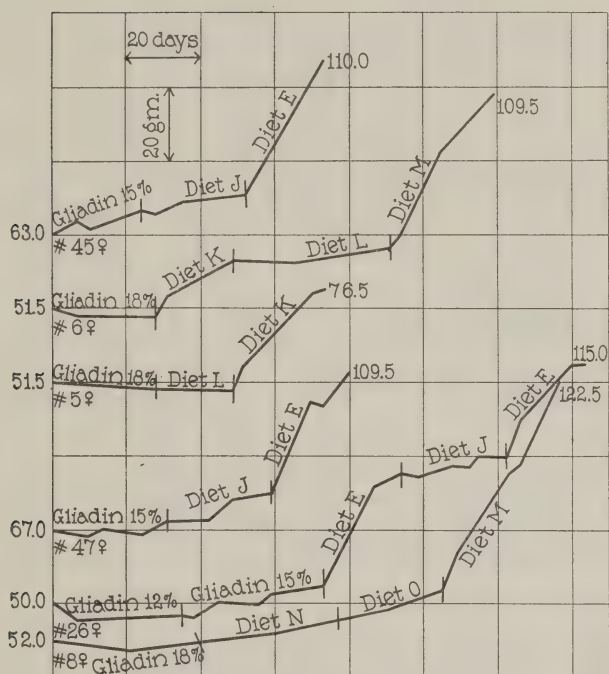
Diet E: Gliadin 15 per cent + lysine $\frac{1}{2}$ per cent.

" S: " 15 " " + ϵ -hydroxycaproic acid 1 per cent.

" T: " 15 " " + synthetic *dl*-lysine 1 " "

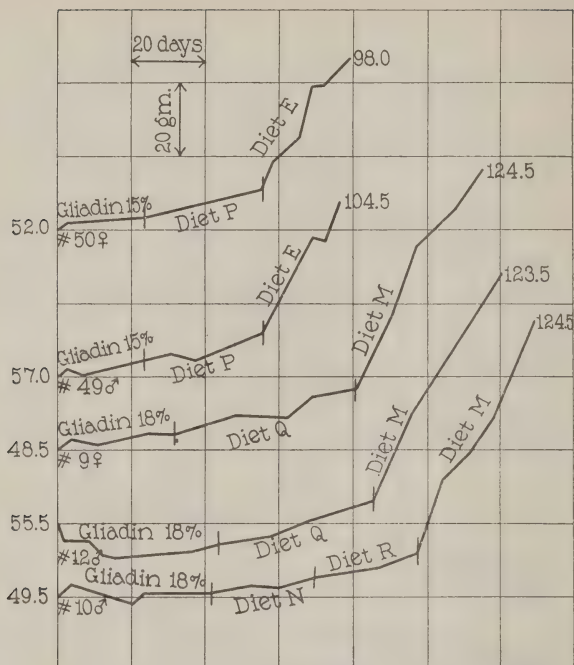
" U: " 15 " " + " " $\frac{1}{2}$ " "

CHART IV. Showing the failure of ϵ -hydroxycaproic acid (Diet S) to supplement gliadin and the response when it was replaced by lysine (Diet E). Curves 66 and 67 show the growth of rats on diets of 15 per cent gliadin plus 1 per cent *dl*-lysine (Diet T) and the response when this diet is replaced by 15 per cent gliadin plus 0.5 per cent *d*-lysine (Diet E). The failure of growth of Rat 67 on 0.5 per cent *d*-lysine is ascribed to a diminished food intake. Curve 68 shows the growth on 0.5 per cent *d*-lysine (Diet U) and the subsequent growth on 0.5 per cent *d*-lysine. Growth on the latter diet is somewhat greater. Compare also Rat 17, Chart III.



Diet E: Gliadin 15 per cent + lysine $\frac{1}{2}$ per cent.
 " J: " 15 " " + ϵ -aminocaproic acid 1 per cent,
 " K: " 18 " " + lysine (?) 1 per cent.
 " L: " 18 " " + ϵ -aminocaproic acid 1 per cent.
 " M: " 18 " " + lysine $\frac{1}{2}$ per cent.
 " N: " 18 " " + cystine 1 " "
 " O: " 18 " " + " 1 " " + ϵ -aminocaproic
 acid 1 per cent.

CHART V. Showing that the rate of growth is not altered when ϵ -amino-caproic acid supplemented the gliadin of the diet at levels of 15 and 18 per cent of protein intake, and the failure to utilize this acid in place of lysine.



Diet E: Gliadin 15 per cent + lysine $\frac{1}{2}$ per cent.

" M: " 18 " " + " $\frac{1}{2}$ " "

" N: " 18 " " + cystine 1 " "

" P: " 15 " " + α -hydroxy- ϵ -aminocaproic acid 1 per cent.

Diet Q: Gliadin 18 per cent + α -hydroxy- ϵ -aminocaproic acid 1 per cent.

Diet R: Gliadin 18 per cent + α -hydroxy- ϵ -aminocaproic acid 1 per cent + cystine 1 per cent.

CHART VI. Showing the effect of adding α -hydroxy- ϵ -aminocaproic acid to gliadin diets. This derivative, even though very closely related to lysine, fails to influence the rate of growth as does lysine, thus demonstrating failure to aminize the α -hydroxy groups even under stress. Substitution of lysine (Diet M) for the other caproic acid derivative increased the rate of growth in all cases. Addition of cystine (Diet R) did not facilitate lysine synthesis from α -hydroxy- ϵ -aminocaproic acid.

THE EFFECTS OF ANOXEMIA ON NITROGEN METABOLISM.

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One of the great problems of biology consists of the determination of the response of the living organism to definite changes in its environment. From the chemical standpoint, the environment of the cells of the body is altered whenever an active pharmacological agent is introduced into the body. Such changes caused by the introduction of a new substance may be referred to as a *positive* change in the chemical environment. The effects of many such positive changes in the chemical environment on metabolism have been most carefully studied. When one withdraws from the environment of the cells some substance to which the cells are accustomed and which is not entirely inert, there is produced an equally vital though *negative* change in the chemical environment. Thus, in a patient accustomed to morphine, one should expect to find that the withholding of the drug would produce certain withdrawal symptoms, and that accompanying these one would expect to find very definite changes in the chemical processes within the cells responsible for the symptoms. This situation is quite analogous to the withdrawal of the active constituent of the thyroid which produces well known changes in metabolism, and many similar instances are to be noted in the so called "deficiency diseases."

Since apparently all the energy required for life is derived from processes of oxidation, every phase of that subject should be studied in great detail. The relation of the supply of oxygen to the rate of oxidation within the body has always interested investigators. Voit (1) showed that the metabolism is not pro-

portional to the oxygen supply. Pflüger (2) came to the same conclusion. The fact that an increase in the oxygen supply does not alter the normal metabolism, has been confirmed in recent years, using modern methods (3).

While it must be regarded as proved, that increasing the oxygen in the respired air at atmospheric pressure does not significantly increase physiological oxidation, it is difficult to understand how any one could maintain that a sufficient reduction of oxygen in the respired air could be without profound effect on the chemical processes of the cells of the body and, therefore, also on their functional activity.

Mosso (4) believed that a reduction of the normal carbon dioxide content of the cell could produce marked changes in functional activity and termed this condition "acapnia." Yandell Henderson (5) strongly supported and extended Mosso's view but is unwilling to admit that a reduction of the oxygen supply to the cells can of itself cause profound changes. Haggard and Henderson (6) state that the view of Gasser and Loevenhart (7) that oxygen deficiency is itself a stimulus to the respiratory center is unsatisfactory because it "leaves the question as to how it does it quite unanswered." Henderson agrees that lessening the CO_2 in the blood and tissues leads to alteration in functional activity. He accepts this because CO_2 alters the pH of the blood and he can apparently accept without difficulty that this somehow leads to alteration of function. The question immediately presents itself, however,—how does the change in the acid-base equilibrium alter function? This question, no one has answered and but few have even asked. It must be granted that the function of a cell must be merely the external expression of the chemical processes going on within the cell and a necessary and automatic concomitant of the state of chemical activities within the cell. The exact mechanism of stimulation by oxygen want cannot be answered until we know much more of the chemistry of living cells than is available at the present time. Loevenhart (8), however, has attempted to form a conception of a possible mechanism. Henderson seems to overlook the fact that if his respiratory X were found to be a substance he would still have to explain how respiratory X stimulates the respiratory center, just as he demands to know how oxygen want stimulates.*

It is certain that X would have to stimulate by altering in some manner the fundamental chemical processes of the cell just as oxygen want does. He would have to show how it affects these processes. Oxygen want certainly affects biological processes. In other words, Henderson prefers to make two hypotheses rather than one. First, he assumes a substance which he can only define negatively, as not being of a strongly acid nature, and then assumes that it so modifies the chemical processes of the cells of the respiratory center as to result in increased functional activity. In our opinion, Henderson's respiratory X is oxygen want.

When one recalls that the most fundamental requirement of life is the transformation of energy, it seems almost axiomatic that any interference with this rendering kinetic of chemical energy can immediately and of itself cause any degree of abnormal functioning up to death of the cell. Great importance attaches to the whole question of the alteration of metabolism as a result of decreased oxidation within the cells. Death in the last analysis is always asphyxial whether the death be due to failure of the respiration or circulation. Therapeutically, it is most important because acute asphyxiation either results in death or complete recovery within a very few minutes and the most intelligent methods of resuscitation will wait upon a complete understanding of the forces at work, both physical and chemical. When the importance of the subject is considered, it is remarkable that more work has not been done to determine the effect of decreased oxidation on metabolism.

In 1882, Penzoldt and Fleischer (9) concluded that the decomposition of body substance is increased by oxygen want. Fränkel and Geppert (10) report studies of the nitrogen metabolism of two dogs exposed to air at a pressure of 200 to 260 mm. of Hg for 7 hours and found that on the day of exposure the nitrogen excretion was not affected. The following days, however, there was a marked increase in the nitrogen excreted in the urine amounting to 75 per cent in one experiment and 60 per cent in the other. In 1896 von Terray (11) found that in dogs and rabbits, the metabolism is independent of the oxygen content of the air between 87 and 10.5 per cent oxygen. When the oxygen of the respired air is reduced somewhat below 10.5 per

cent and down to 5 per cent, there occur manifestations of oxygen want in the tissues, the most striking being an increase in the excretion of carbon dioxide, a smaller rise in the nitrogen excreted, and a rise in the respiratory quotient. The oxygen absorption falls with the increased elimination of carbon dioxide, both effects being the more marked the greater the lowering of the oxygen of the respired air. At 5.25 per cent oxygen the great majority of animals show an increased excretion of nitrogen. He stated that in spite of a considerable reduction of oxygen absorbed, the decomposition processes are increased. He noted that there is a marked production of organic acids and concluded that oxygen want causes both quantitative and qualitative changes in the metabolism. At very low oxygen percentages he found the urine to be usually decreased in amount and to contain albumin but no sugar.

Many other studies have been made to determine the effect of reduced barometric pressure, either on high mountains or in pneumatic chambers, on the nitrogen metabolism. These investigations, however, were for the purpose of elucidating the problems of high altitude and the nature of the physical adaptation or acclimatization to high altitude rather than the fundamental proposition of determining what happens chemically when the cells of higher animals are allowed only the minimal supply of oxygen just compatible with life. It is only by such means that we might hope to determine to what extent the cells of the higher forms may derive energy from anaerobic processes and what type of processes are involved.

Thus, the work of Durig (12), von Wendt (13), Hasselbalch and Lindhard (14), and Sundstroem (15) does not elucidate this phase of the problem because the degree of oxygen want was not sufficiently extreme. In some instances, these investigators found a positive and, in others, a negative nitrogen balance, while in still others, no effect was observed. The effect of hemorrhage on metabolism has been studied by several investigators and they have found an increase of nitrogen eliminated in the urine (16).

In studying the effect of decreased oxidation on metabolism, it is important to know what changes are produced simultaneously in the acid-base equilibrium of the blood and tissues.

Since changes in the pH of the blood induced by oxygen want will form the subject matter of two other communications, it will be touched upon only sufficiently to make the present article more intelligible. Araki (17) and Zillessen (18) showed that decreased oxidation leads to the production and excretion of lactic acid, and it has been assumed ever since that this results in acidosis. There has been excellent experimental evidence of the production of the organic acids under various conditions that would interfere with oxidation, but proof has never been furnished, as far as we are aware, that there is an actual increase in the hydrogen ion concentration of the blood as a result simply of decreased oxidation as brought about by a diminished supply of oxygen. Haggard and Henderson (6) have found that oxygen want produces not acidosis but alkalosis. This they very properly explain as due to the loss of carbon dioxide incident to the hyperpnea produced by oxygen want. This fact lends support to the view of Loevenhart (7, 19) that oxygen want *per se* stimulates the respiration without the intervention of acid bodies. In their paper entitled "The fallacy of asphyxial acidosis," Haggard and Henderson claim that the theory of asphyxial acidosis is not in accord with the facts which they bring out. Subsequent papers from this laboratory will bring definite proof of the fact that the effect of anoxemia on the acid-base equilibrium of the blood is diphasic, causing first alkalosis, then acidosis. Alkalosis does not markedly alter lung ventilation nor increase the formation and excretion of ammonia, while acidosis should increase them. A slight degree of alkalosis should likewise be expected rather to inhibit the breakdown of protein and lessen the total nitrogen excretion and acidosis might be expected to have the opposite effect since it is known that a change toward the acid side so greatly favors autolysis *in vitro* (20). Furthermore, if acidosis favors autolysis of muscular tissue, one should expect to find an increase in the creatinine-creatine excretion.

Koehler, Brunquist, and Loevenhart (21) have shown that as a result of breathing air deficient in oxygen, there first results an alkalosis which is followed by a return to the normal pH of the blood. Then, if the oxygen want is sufficiently severe, there develops a severe acidosis. These changes and the time relationships appear in detail in the other papers referred to. We de-

terminated to study the relation of reduced oxidation to metabolism, using modern methods and following simultaneously the changes in the hydrogen ion concentration by the electrometric method. The pH determinations were made by Dr. A. E. Koehler to whom our thanks are due. We fully realize that the work here reported is very incomplete and that much more work on this subject remains to be done. Thus, the results of von Terray regarding the elimination of carbon dioxide and oxygen absorption should be confirmed. An effort should be made to determine the nature of the organic acids produced and the mechanism by which they may be so rapidly produced and equally rapidly destroyed under certain conditions. It is hoped to continue work along this field in this laboratory, but in view of its interest and importance we hope that work along similar lines will also be undertaken elsewhere.

EXPERIMENTAL METHODS.

Seven experiments upon pigs and one on rabbits are reported here. The pigs used were very young, having only been weaned a short time and weighing from 9 to 17 kilos. The reason for selecting young pigs was that the size of our respiratory chamber limited the size of the animal that could be used. The very active metabolism of young animals seemed favorable to the work. The animals were exposed at atmospheric pressure to atmospheres low in oxygen content with a correspondingly high nitrogen content. The respiratory chamber used was essentially like that described by Kolls and Loevenhart (22), but somewhat larger and heavier, with additions and modifications described by Seybold and coworkers (23). The oxygen content of the atmosphere of the chamber was lowered by means of the device described by Kolls and Loevenhart for burning hydrogen. This was placed in the absorbing circuit only during the time that the oxygen content was being reduced. During this period a small tube in the wall of the chamber was left open to the outside air so that the oxygen absorbed by the hydrogen flame was replaced by air. By this means the oxygen can be reduced to approximately 7 per cent. Two methods were used to lower the oxygen content below the point to which it could be reduced by means of the hydrogen flame. (1) The automatic oxygen

supply was closed and the tube connecting the chamber with the outside air opened. Then, as oxygen was used by the animals, its place was taken by air and the oxygen content of the chamber was gradually lowered. (2) In case a more rapid lowering of the oxygen content was desired, hydrogen in the absence of nitrogen was used to replace a part of the atmosphere of the chamber. In most experiments the pigs showed marked response to lowering of the oxygen content to 9 per cent and the oxygen level was not further reduced immediately. While we desired to submit the animals to as severe a strain as they could withstand, we had to reckon with vomiting which would contaminate the urine and thus spoil the experiment. Furthermore, if the strain of oxygen want were too great, the animals secreted little or no urine. A few of the pigs died after a few hours on being suddenly subjected to an oxygen content of 8 to 9 per cent. In many cases, therefore, we held this atmosphere for a sufficient period of time to determine the response of the animal and to allow some time for adjustment before further reduction.

In determining the suddenness and extent to which we could lower the oxygen content, we had to be governed by the behavior of each animal. Thus, the respiratory rate, severity of dyspnea, posture, and general behavior were carefully noted in order to avoid any of the mishaps which would spoil the experiment. If the lowering of the oxygen content were not of sufficient degree or suddenness, the various compensating factors in the circulation, respiration, and bone marrow would come into play and little or no effect upon the metabolism would be noted. For these reasons many of our experiments were failures. In some cases, the animal was not subjected to sufficient strain and little or no effect on the metabolism was noted. In other cases vomiting, suppression of secretion of urine, or death occurred.

In order to secure thorough mixing of the air in the chamber, an 8 inch electric fan was placed in it. The motor operating this fan was outside the chamber and the shaft of the fan passed through a tight stuffing box. This was necessary because in some experiments the oxygen content of the chamber was lowered by displacing part of the atmosphere with hydrogen. Hence, it was possible that at times there would be an explosive mixture of oxygen, hydrogen, and nitrogen in the chamber and sparks

from the motor would be dangerous. The oxygen content was determined many times during the day and night by means of the phosphorus pipette. The carbon dioxide content was likewise determined, using the Haldane apparatus. The carbon dioxide content was always kept so low by the use and frequent renewal of large soda-lime absorbers that it was no factor in the experiments.

With regard to diet, it was found that the pigs would not eat when subjected to low oxygen. Therefore, it was necessary to have a period of fasting in an atmosphere of ordinary oxygen content to compare with the period of fasting at low oxygen content. In order to control the situation more accurately, however, the pigs were placed on a standard diet, previous to the experiments, at a little above maintenance for these young animals. The animals received 80 calories per kilo per day, which included 0.43 gm. of nitrogen.

The diet per kilo was as follows:

	<i>gm.</i>	<i>cals.</i>
Casein.....	3.68	(14.7)
Fat.....	2.38	(22.4)
Starch.....	10.7	(42.8)

In addition, they received the following salts.

Sodium chloride.....	0.04 gm.
Potassium chloride.....	0.04 "
Calcium acid phosphate.....	0.1 "
Magnesium sulfate.....	0.01 "
Sodium carbonate.....	0.25 "
" acetate.....	0.25 "
" citrate.....	0.25 "
Water to.....	70 cc.

This diet yielded firm solid feces and enabled us to collect the urine uncontaminated with fecal matter. It was found necessary, in order to keep the animals in good condition and to avoid dietary acidosis, to include the sodium carbonate, acetate, and citrate, as mentioned above.

The pigs were placed upon the standard diet for a few days before examinations of the urine were started. Then, the metabolism of the animals was followed for a period of 3 or 4 days. The animals were then placed on a fasting diet in an atmosphere of

normal oxygen content for a period of 2 to 4 days, during which time metabolism was studied. Then there was a rest period of approximately 4 days on the standard diet followed by various periods of fasting at low oxygen. In Table I we show three periods: (1) the average 24 hour excretion of each of the urinary constituents studied at normal oxygen and standard diet; (2) the average 24 hour excretion under normal oxygen and fasting; and (3) the average 24 hour excretion at reduced oxygen and fasting. The animals were frequently weighed during the experiments. They were all kept in the same room during all of the periods. This room was kept at a fairly constant temperature and all of the conditions except the oxygen content of the air breathed were constant.

With regard to the rabbits, it is obvious on account of the prolonged storage of food in these animals that there would be no point in placing them on a carefully controlled diet for brief periods. Here several animals were placed in the chamber at the same time and the composite urine was analyzed. The urinary constituents on the ordinary laboratory diet were first determined, then food was withheld during a period at ordinary oxygen for comparison with a similar period of fasting at low oxygen concentrations.

The flask in which the urine was collected was closed to the outside air and toluene was kept in it. All analyses were made at the end of each 24 hour period and the averages of the daily excretion are given in Tables I and II. On account of urine remaining in the bladder from the previous day, the average excretion for each period is much more significant than the daily voidings. The carbon dioxide and hydrogen ion content of the blood was determined separately for each animal, both in the rabbit and pig experiments, but the averages only are given in the rabbit experiment.

The analytical methods used in the work were as follows:

Total nitrogen...Folin's microchemical method (24).

Urea...Marshall's urease method.

Ammonia...Folin's aeration method (24).

Creatinine and creatine...Folin's microcolorimetric method (24).

Total acidity...Folin's titration method (24).

Total organic acids...Van Slyke and Palmer's titration method (25).

TABLE I.—*Analysis.*24 hour averages for periods, respectively, of: (a) Normal O₂ tension, with diet (standard diet)

Experiment.	Weight.	Conditions.	Length of period.	Range of O ₂ content.	Amount of urine.	Total nitrogen.	Urea nitrogen.
	kg.		hrs.	per cent	cc.	gm.	gm. per cent
Fig 1.	10.5	Normal O ₂ and diet.	96		1,383	2.324	1.571 67.
	11.2	“ “ “ fasting.	48		1,830	2.332	1.697 72.
	10.8	Reduced “ “ “	46	10.0 to 7.9	341	2.675	1.99 74.
Fig 3 (first experiment).	16.8	Normal O ₂ and diet.	96		442	3.315	1.698 51.
	16.2	“ “ “ fasting.	96		212	2.12	1.204 56.
	14.3	Reduced “ “ “	96	10.2 to 4.0	293	6.335	2.354 37.
Fig 5 (first experiment).	9.0	Normal O ₂ and diet.	72		397	1.453	0.529 36.
	8.5	“ “ “ fasting.	72		263	2.424	1.364 56.
	7.72	Reduced “ “ “	110	16.7 to 5.2	211	2.650	1.317 49.
Fig 6.*	13.4	Normal O ₂ and diet.	96		341	2.722	1.923 70.
	11.82	“ “ “ fasting.	96		219	2.332	1.192 51.
	12.82	Reduced “ “ “	66	7.7 to 7.5	279	3.614	2.397 66.
Fig 3* (second experiment).	16.5	Normal O ₂ and diet.	48		502	2.697	1.218 45.
	16.6	“ “ “ fasting.	96		212	2.120	1.204 56.
	15.3	Reduced “ “ “	70	8.3 to 6.9	307	4.366	2.107 48.
Fig 5 (second experiment).	9.38	Normal O ₂ and diet.	72		382	2.361	1.470 62.
	8.5	“ “ “ fasting.	72		263	2.424	1.364 56.
		Reduced “ “ “	96	8.5 to 6.2	110	2.415	1.113 46.
Fig 7.	9.5	Normal O ₂ and diet.	72		456	2.720	1.440 52.
	9.2	“ “ “ fasting.	48		300	2.211	1.070 48.
	8.8	Reduced “ “ “	42	8.5 to 7.7	250	3.206	1.909 59.
Three rabbits.	7.47	Normal O ₂ and diet.	72		147	3.56	1.61 45.
		“ “ “ fasting.	48		250	3.477	1.568 45.
		Reduced “ “ “	114	7.5 to 5.5	202	3.41	1.84 54.

* Figs 3 and 6 died at the end of the experiments.

DISCUSSION.

In the following discussion, the term “normal” will be used to designate the period wherein the animals were on the standard

Urine.

case of pigs); (b) normal O₂ tension, with fasting; and (c) reduced O₂ tension, with fasting.

Ammonia nitrogen.		Creatinine nitrogen.		Creatine nitrogen.		Creatinine and creatine nitrogen.		Organic acids.	Acidity by titra- tion.	Blood CO ₂ .	Blood pH.
gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	cc. 0.1 N	cc. 0.1 N	vol. per cent	
339	14.6	0.152	6.5	0.066	2.8	0.218	9.4	127	50.8	55.8	7.403
313	13.4	0.107	4.6	0.041	1.8	0.148	6.4	151	55.4	55.7	7.428
099	3.7	0.119	4.4	0.067	2.5	0.186	6.9	145.4	28.3	37	7.480
680	20.5	0.144	4.3	0.038	1.1	0.181	5.4	310.5	69.0	53.3	7.386
348	16.4	0.127	6.0	0.047	2.2	0.174	8.2	194.4	68.4	47.3	7.417
840	13.3	0.094	1.5	0.116	1.8	0.210	3.3	442.6	71.1	24.5	7.269
357	24.5	0.099	6.8	0.029	2.0	0.128	8.8	56.5	148.6	38.1	7.401
256	11.4	0.091	3.7	0.036	1.5	0.127	5.2	68.2	104.4	56.2	7.390
152	5.7	0.075	2.8	0.060	2.3	0.136	5.1	200.7	85.8	53.8	7.350
387	14.2	0.136	5.0	0.027	1.0	0.163	6.0	199.6	61.1		
156	6.7	0.090	3.9	0.049	2.1	0.139	6.0	136.5	97.6		
483	13.4	0.107	2.9	0.118	3.3	0.224	6.2	519.8	144.4		
505	18.7	0.127	4.7	0.030	1.1	0.157	5.8	337.9	48.6	54.3	7.443
348	16.4	0.127	6.0	0.047	2.2	0.174	8.2	194.4	68.4		
518	11.9	0.137	3.1	0.141	3.2	0.278	6.3	471.1	119.7	11.8	6.901
375	15.9	0.096	4.1	0.020	0.8	0.116	4.9	161.4	47.9	45.6	7.432
256	11.4	0.091	3.7	0.036	1.5	0.127	5.2	68.2	104.4	56.2	7.390
382	15.8	0.074	3.0	0.041	1.7	0.114	4.7	137.8	27.2	24.4	7.201
295	10.8	0.125	4.6	0.048	1.8	0.173	6.4	133.2	78.1	48.4	7.421
268	12.1	0.081	3.7	0.058	2.6	0.139	6.3	125.2	70.2		
129	4.0	0.116	3.6	0.080	2.5	0.196	6.1	165.4	103.2	44.0	7.340
014	0.4	0.075	2.1	0.047	1.3	0.122	3.4	210.2	104.4	48.34	7.476
010	0.3	0.099	2.9	0.037	1.1	0.136	4.0	110.8	100.7	35.15	7.378
017	0.5	0.103	3.0	0.083	2.4	0.186	5.4	227.2	96.0	20.0	7.273

diet at normal oxygen tension, the term "fasting" is applied when they were fasting in an atmosphere of normal oxygen content, and "anoxemia" the period when they were fasting at lowered oxygen tension. The significant comparison is to be

TABLE II.
Percentage Variations in Urinary Metabolites Incident to Reduced Oxygen Tension.

Experiment.	Dura- tion of anox- emia, hrs.	Range of O ₂ content, per cent	pH of blood at close of anoxemia.	Total nitrogen, per cent	Urea nitrogen, per cent	Am- monia nitrogen, per cent	Creati- nine nitrogen, per cent	Creatine nitrogen, per cent	Creatinine and creatine nitrogen, per cent	Organic acids, per cent	Acidity by titration, per cent
Pig 1.	46	10 to 7.9	7.48	+15	+17	-68	+11	+64	+26	-4	-49
" 3.	96	10.2 to 4.0	7.26	+199	+96	+141	-26	+147	+21	+128	+4
" 5.*	110	16.7 to 5.2	7.35	+9	-3	-41	-17	+67	+7	+194	-18
" 6.†	66	7.7 to 7.5		+55	+101	+210	+19	+141	+62	+281	+48
" 3† (second ex- periment).	70	8.3 to 6.9	6.9	+106	+75	+49	+7	+201	+60	+142	+75
Pig 5 (second ex- periment).	97	8.5 to 6.2	7.2	-0	-18	+49	-19	+12	-10	+102	-74
Pig 7.	43	8.5 to 7.7	7.34	+45	+78	-52	+43	+38	+41	+32	+47
Three rabbits.	114	7.5 to 5.5	7.27 (Average)	-2	+17	+71	+4	+124	+36	+105	-5

* Experiment practically equivalent to two short experiments (see text).

† Pigs 6 and 3 died at conclusion of experiments.

made between the fasting and anoxemia periods. The period under standard diet and normal oxygen is given merely to indicate the condition of the metabolism under more normal conditions in the animals with which we were working. Table I gives the conditions of the experiments and the analytic findings. Table II gives the alterations in the metabolism of the fasting animal incident to exposure to atmospheres of low oxygen content.

A brief statement regarding each experiment will facilitate the understanding of the results.

Pig 1.—This was a rather short exposure to an atmosphere of hardly low enough oxygen content to produce a definite change in metabolism. The oxygen content was higher than was allowed in any other experiment. Henderson and Haggard were the first to show that mild degrees of anoxemia result in alkalosis and the blood pH in this case shows that at the end of the experiment there was a mild degree of alkalosis. In the light of other results which we have obtained, it is probable that the degree of alkalosis had been greater somewhat earlier in the experiment and that the pH was in process in swinging to the acid side as evidenced by the low CO_2 content of the blood. Under conditions of alkalosis we should not expect an increase in nitrogen elimination. The reduction in the ammonia excretion was definite and apparently is to be regarded as a response to the alkalosis.

Pig 3 (First Experiment).—This is the best experiment, all points considered, of the entire series. In this animal we happened to strike the optimum conditions for showing the effect of anoxemia on metabolism by the methods used. Figs. 1 and 2 show graphically the effect of anoxemia on the nitrogen metabolism and the blood reaction in this experiment.

Pig 5 (First Experiment).—This experiment was faulty in that through an error the oxygen was allowed to rise to 16 per cent on the 3rd day and remained above 12 per cent for 9 hours. This experiment was therefore equivalent to two 48 hour experiments with an interval of over 12 hours at an oxygen level which does not alter the normal metabolism in these animals and which favored a return to normal metabolism.

Pig 6.—This animal died in the respiratory chamber and death was not discovered until 2 hours afterward so that the final figures of the blood CO_2 and pH could not be obtained.

Pig 3 (Second Experiment).—This experiment was performed within a month of the first experiment on this animal. The animal was not submitted to fasting at normal oxygen a second time so the figures for this period are the same as those in the first experiment on the animal. The greatly reduced volume of urine excreted in this case is very striking and the failure of this animal to show an increased output of nitrogen is in all probability due to renal embarrassment incident to the anoxemia. Except for the decrease in volume the urine during the low oxygen run shows re-

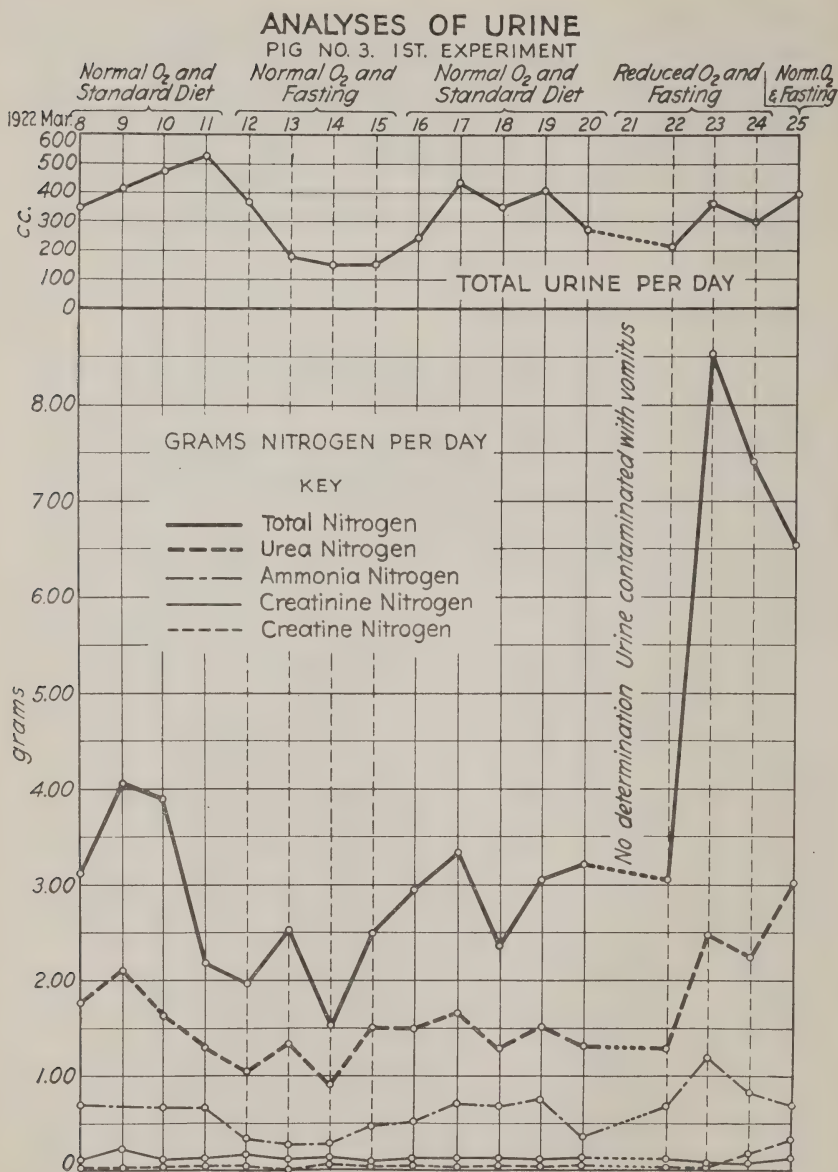
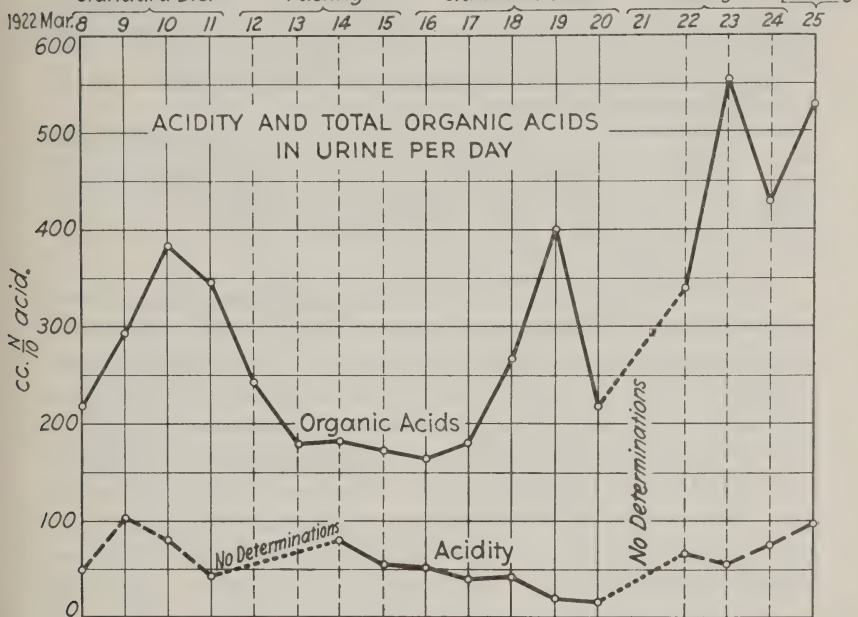


Fig. 1.

ANALYSES OF URINE

PIG NO. 3. 1ST. EXPERIMENT

Normal O₂ and
Standard DietNormal O₂ and
FastingNormal O₂ and
Standard DietReduced O₂ and
FastingNorm. O₂
& Fasting

ANALYSES OF BLOOD

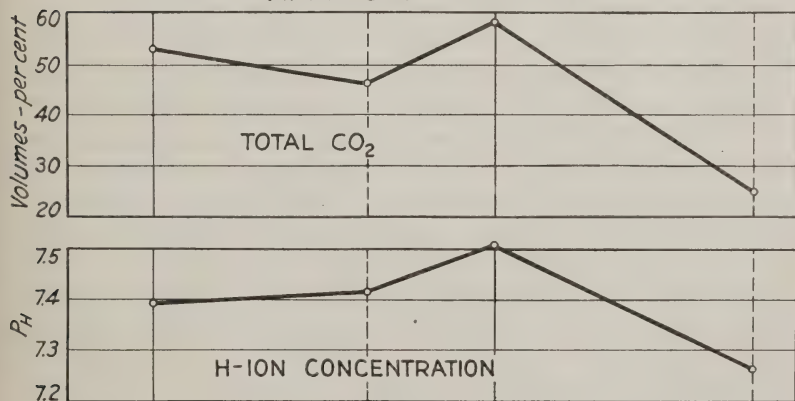


Fig. 2.

markably little alteration from the normal. Blood analyses in this case would probably have shown that the kidney derangement was largely responsible for this. This animal died at the end of the experiment. The autopsy findings in Pigs 3 and 6 agree closely with the morphological changes described by Martin, Loevenhart, and Bunting in rabbits as a result of exposure to atmospheres of low oxygen content (26).

Pig 7.—This was an average experiment of relatively short duration. The animal showed no acidosis.

Three Rabbits.—In this experiment we started with six rabbits, three of which died in the course of the anoxemia. The composite urine was analyzed. The blood CO_2 and pH were determined in each individual and the results were strikingly uniform in these determinations. The average figures alone are given.

Total Nitrogen.—The total nitrogen excretion was increased in low oxygen in all experiments except Pig 5 (second experiment) and in the rabbit experiment, and in these two it was practically unaltered. In the case of Pig 5 (second experiment) the great decrease in the volume of urine excreted, almost certainly due to renal involvement incident to anoxemia, satisfactorily accounts for the failure to show an increase. Martin, Loevenhart, and Bunting (26) showed that the kidney of rabbits submitted to low oxygen tension shows swelling of the cells of the cortical tubules until the tubular lumen is almost obliterated. We have no satisfactory explanation to offer for the fact that in the case of the rabbits, no increase in nitrogen excretion was observed. Possibly this too may have been due to abnormal kidney function, but the volume of the urine excreted was not reduced. The increase in nitrogen excretion was quite small in Pigs 1 and 5 (first experiment). In the former the oxygen tension was too high to produce much effect and in the latter there was a rise in the oxygen almost to normal level during the experiment, as already explained. The increase in the nitrogen excretion is striking in the remaining experiments; *viz.*, Pigs 3, 6, and 3 (second experiment). Our results agree, therefore, with those of Penzoldt and Fleischer, Fränkel and Geppert, and von Terray that oxygen want of sufficient grade increases nitrogen metabolism.

What is the mechanism and significance of the increased nitrogen excretion on exposure to atmosphere of low oxygen content? From our standpoint one can view it more clearly if we assume that the most urgent factor in the continuance of the life of

cells is that stored chemical energy shall be continuously rendered kinetic. Any factor that interferes with the processes concerned will cause deep seated changes in the chemical status of the cell and in its functional activity. Several contributions from this laboratory have sought to elucidate the relation between changes in the oxidative processes within cells and their state of functional activity. Interference with the transformation of energy within the cells of the higher form may be brought about in many ways: (1) interference with external respiration; (2) interference with the oxygen-carrying power of the blood; (3) interference by physical or chemical means with oxidative processes within the cells; (4) by fasting so that there results a lack of material to be oxidized; and (5) a lack of oxygen.

In regard to the latter, we know that many living forms have the power so to alter their chemical processes that they can live under anaerobic conditions. In this way they can derive enough energy for their meager needs without having any free oxygen available. One might, therefore, expect all cells to have power more or less to adapt their chemical processes to a reduction in the supply of oxygen. Increased nitrogen breakdown might then be considered as an adaptation to anoxemia, possibly yielding more energy per gram of oxygen required. Whether this is the case or not, we are confronted with the question as to the mechanism by which it is brought about. We have indicated in Tables I and II the pH of the blood at the conclusion of anoxemia which clearly proves that in certain of the experiments there existed an acidosis. From the tables it will be seen that in most instances there is a relation between the degree of acidosis and the increase in the nitrogen elimination.

In the experiment with Fig 1 there was an alkalosis and but a slight increase in the nitrogen excretion. Fig 5 (first experiment) showed a normal pH and again there is but little change in the nitrogen excretion. Fig 7 is an exception and with a normal pH there is a definite increase. On the other hand, the rabbits showed no increased nitrogen elimination in spite of a definite acidosis. While the same is true of Fig 5 (second experiment), here we believe the explanation of renal embarrassment explains this instance. There could be only a general relationship between the blood pH and the nitrogen elimination because

with a sufficient and sudden lowering of the blood pH, the function of the kidneys becomes much impaired. In other words, the relation could only hold within a narrow range of blood pH. The most striking increase in nitrogen elimination was in Fig 3 (first experiment) which showed only a moderate degree of acidosis. The sensitivity of the kidneys of different animals to oxygen want varied markedly. Thus, Fig 1 showed a striking decrease in the volume of urine secreted at a relatively high oxygen exposure and in spite of the fact that there was no acidosis. It is probable that with acidosis there always occurs an increase in the protein metabolism, but in order to prove this it would be necessary to have blood analyses in case the urine, as a result of abnormal kidney function, did not show an increase in nitrogen elimination.

It is well known that autolysis *in vitro* occurs much more rapidly and completely when the hydrogen ion concentration is increased (20). It seems, therefore, that the mechanism of increased nitrogen elimination may be the shifting of the acid-base equilibrium toward the acid side as suggested by Schryver (27). It would seem also that this is the probable mechanism of the premortal rise in nitrogen metabolism noted in fasting; namely, with the increasing acidosis of fasting there is an increase in tissue autolysis. It would seem that fasting as well as a decreased supply of oxygen results in acidosis with increased autolysis and nitrogen elimination, and since both result in interference with energy transformation it would seem that the alteration of the chemical processes in cells from either of these causes is quite similar.

There has been considerable discussion of the cause of death in fasting and in asphyxia. Acidosis and the accumulation of toxic products due to incomplete degradation or anaerobic cleavage products have been assigned to the rôle of the immediate lethal agent. However, no investigator has attempted to say exactly how these hypothetical toxic products kill. Death will not occur until the cell as a mechanism for transforming chemical energy becomes too inefficient to support life. This, it seems to us, is the essential thing. Death from asphyxia may be almost instantaneous as in the case of an overwhelming dose of hydrocyanic acid injected intravenously. Death occurs too

quickly to assume the intermediate production of toxic products. Are we to assume here anything other than that the energy-yielding processes are reduced below the minimum required to support life? It seems to us that the subject becomes somewhat simpler when viewed from the standpoint of energy. It does not clarify matters to introduce a hypothetical intermediary mechanism and then to give no suggestion as to how it may act. It is like assuming a poisoning of the furnace when the fire goes out either from the lack of fuel or of oxygen. It is true that abnormal products, such as carbon monoxide, may be produced as the fire goes out but we need not assume that in some unknown way they extinguish the fire. The fire goes out when the temperature of the furnace drops below the ignition point of coal, although there may be some coal and some oxygen left. This view does not lessen our interest in the character of the products of the nature of the chemical transformations occurring in cells under conditions of fasting or oxygen want or the study of the mechanism by which these chemical changes are brought about.

Table II shows that the excretion of urea nearly always follows changes in the total nitrogen as a result of oxygen want. In some experiments the increase in urea nitrogen excretion exceeds that of the total nitrogen and in others it is less. In regard to ammonia excretion, it is interesting to note that in Fig 1, the only animal in the series showing alkalosis, there is the most marked decrease in ammonia excretion found in any of the animals. However, a decrease in ammonia excretion was noted in Fig 5 (first experiment) and in Fig 7, in both of which there was but little change in the blood pH. It is certain that both of these animals were in a condition of alkalosis during most of the experiment because, as pointed out previously, the first experiment on Fig 5 was through an error equal to two short runs and Fig 7 was also a very short run of only 43 hours. Table I shows that the percentage of total nitrogen excreted as urea in the best experiment tends to decrease. There is no noteworthy change in the percentage of total nitrogen excreted as ammonia. It seems that the type of acidosis produced by oxygen want does not induce a marked increase in ammonia excretion.

Creatinine and Creatine.—Table I shows that in all the experiments with pigs in which the anoxemia was maintained for

96 hours or more, there was a decrease in the excretion of creatinine. The decrease is by no means striking, regarded alone, but when one considers the marked increase in total nitrogen excretion in the longer experiments, the failure of the creatinine to increase is striking. Thus, in Fig 3 (first experiment), the nitrogen excreted under low oxygen was three times as great as under normal oxygen while the excretion of creatinine incident to exposure to low oxygen suffered a loss of 26 per cent.

In considering the results with creatine, we point out again that we were dealing with young animals. It was first shown by Amberg and Morrill (28) that the urine of young infants contains a considerable amount of creatine and their observation has been confirmed many times.

Table II shows that the excretion of creatine increased in every experiment without exception. The maximum is shown by Fig 3 (second experiment) in which the creatine excretion was three times as great under low oxygen as under normal oxygen. In three experiments there was a creatinine-creatine crossing, that is, the excretion of creatine under low oxygen exceeded that of creatinine, as in the cases which show the largest creatine excretion there was in most cases an increase also in the creatinine excretion. The excretion of total creatinine-creatine nitrogen was therefore increased in all experiments except in Fig 1 which showed an alkalosis. Without going into a detailed discussion of the various views held and the very extensive literature regarding the biological significance of creatinine and creatine, the results support the following views. (1) That a disturbance of the acid-base equilibrium toward the acid side favors the appearance of creatine in the urine. (2) That an increased endogenous metabolism leads to an increase in the excretion of these substances. (3) Our work would seem to favor the view that creatine ordinarily is destroyed by oxidation or converted by dehydration to creatinine, but with increased production and conditions which interfere with oxidation it appears in the urine.

The work of Gross and Steenbock (29) suggests that there is a relation between oxidation and creatinuria. They noted that the feeding of sheep thyroid to the pig markedly stimulates creatine excretion. It would seem from our results that this

effect of thyroid feeding can be explained upon the basis of altered oxidation since thyroid feeding while tending to increase oxidation and, therefore, the need for oxygen also renders the animal more susceptible to oxygen want and thus may cause less complete oxidation.¹ This should be favorable to the appearance of creatine in the urine. We would, therefore, attribute the increased excretion of creatine under thyroid feeding and under anoxemia to the increased rate of destruction of protein in the body, together with insufficient oxygen to yield the products of normal and complete oxidation. We must distinguish between reduced oxidation from the standpoint of the amount of oxygen absorbed and carbon dioxide produced and the end-point or final products of oxidation. It is perfectly possible to have an increased amount of oxidation with incomplete oxidation.

Acid Excretion.—In every experiment except Fig 1, showing alkalosis, there is a marked increase in the excretion of organic acids. The titratable acidity of the urine was not correspondingly increased. In two experiments there was practically no change in titratable acidity and in the remainder, those showing an increase and a decrease were about equally divided. We have not determined the nature of these acids. Qualitative tests for lactic acid were positive during the periods of anoxemia in all experiments, whereas they were uniformly negative during fasting at normal oxygen. With one exception, acetone was found in the urine of all pigs, which were kept at low oxygen for 72 hours or longer, and was absent in all cases of fasting at normal oxygen. Neither sugar nor albumin was found in the urine by the ordinary clinical tests.

SUMMARY.

Young pigs were subjected to atmospheres of low oxygen content and the effect of the resulting anoxemia on the nitrogen metabolism, as revealed by analyses of the urine, was studied. These findings were correlated with changes in the acid-base equilibrium of the blood which was followed electrometrically. The following effects on the metabolism were noted in the ani-

¹ Asher, L., personal communication.

mals which showed acidosis as a result of the anoxemia. (1) Increase in the total nitrogen excretion; (2) increase in urea excretion which is usually not so marked as the total nitrogen; (3) increase in the ammonia excretion (one pig which showed an alkalosis showed a marked reduction in the ammonia excretion); (4) creatinine excretion is not decidedly or uniformly affected; (5) increased creatine excretion in every animal, which in three cases exceeded the creatinine excretion; and (6) increased excretion of organic acids.

The significance of these results is discussed.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

V. THE EFFECT OF LIGHT UPON CALCIUM AND PHOSPHORUS EQUILIBRIUM IN MATURE LACTATING ANIMALS.*

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A number of investigators have studied the relation of light, as an equivalent or supplement, to the antirachitic factor. These studies have involved the use of light in the prevention of rickets and the inducement of normal bone formation (1); in its relation to the maintenance of a normal calcium and inorganic phosphorus content of the blood (2); and in its relation to growth (3). In all of this work it has been shown that certain wave-lengths of the spectrum are able to duplicate in effect the results secured through the administration of such antirachitic foodstuffs as cod liver oil, green plant tissue, or egg yolks. Obviously if light is able to produce such effects on calcium and phosphorus deposition in cases of young growing animals, it is logical to inquire into its relation to the equilibrium of these elements in the mature animal and particularly the mature lactating animal.

In the young growing animal failure of normal deposition of calcium and phosphorus in osteoid tissue, which is sometimes accompanied by lowered inorganic phosphorus and lowered total calcium in the blood, results in the production of rickets. In the case of the mature animal or in arrested growth where normal bone tissue has already been formed but through adverse dietary or through adverse environmental factors such tissues have suffered a partial depletion of their mineral content—a condition

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known as osteoporosis develops. In rickets there is a failure of normal bone growth due to faulty mineral deposition in the area of proliferation, while in osteoporosis there is a removal of the minerals after deposition of the calcium salts has taken place. One may expect that the two maladies,—rickets and osteoporosis,—are of similar origin and produced by the presence or absence of identical factors,—the only real difference between the two being that one is observed more often in young growing animals while the other condition occurs more frequently in mature animals, with differences between the two cases in the histological picture of the bone tissue due to variation in the extent and place of deposition or resorption of calcium salts. The distinction on the basis of age of the animal is probably not sharp. In its broadest sense rickets or osteoporosis is the result of a failure to assimilate calcium and phosphorus at normal rates. Its effect has been studied mainly in bone tissue, but other tissues are no doubt involved.

Some of the work we have already reported upon confirms this point of view (4); namely, that the same foods which possess antirachitic properties will produce calcium and phosphorus storage in depleted mature animals. Our early work showing the presence of the antirachitic factor in green plant tissue (4) was carried out with mature goats. Later work showing the variation in the antirachitic properties of oat straw, green oats, and dried green oats (5); dried and raw cabbage (5); orange juice and cod liver oil (5); alfalfa hay cured in various ways (6); mixed green grasses (June grass and white clover) supplemented and non-supplemented with calcium salts (7) was done with mature milking goats or mature lactating cows. Some recent experiments by Shipley, Kinney, and McCollum (8) on the antirachitic properties of plant tissue confirm our earlier observations. The method of investigating the effects of light on mature and lactating animals has been to measure the negative or positive calcium and phosphorus balances produced. Such data are quantitative in character and appear to be of high value in studies of this kind when the function of the animal and the level of intake of calcium and phosphorus are given proper consideration. Perhaps it is easier to understand negative calcium balances in osteoporosis than in rickets, the former involving as it must a failure to replace the

calcium waste produced in the metabolism of bone tissue. We have not studied calcium balances in young growing animals known to be suffering from rickets. Possibly in some of these cases positive calcium and phosphorus balances prevail, but the amount of retention in a unit of time would not be a normal one.

The problem of the effect of light upon the balance of calcium and phosphorus in mature animals has considerable practical bearing. It will influence our ideas on the use of minerals in the nutrition of dairy cows; on the avoidance of rickets or osteoporosis in breeding swine; on the use of calcium and phosphorus by the laying hen; in fact, the entire problem of the feeding of farm animals must receive in certain particular aspects new considerations. In addition, if it should be shown that light can play an important part in the maintenance of calcium and phosphorus equilibrium in mature animals a reinterpretation of old experiments, including some of our own, will have to be made.

EXPERIMENTAL.

For this work we have used two mature goats, *milking* during part of the experiment, and one mature *non-milking* goat; both milking goats freshening during the progress of the experiment and before they were subjected to the influence of ultra-violet light.

The ration fed was one carrying a liberal supply of calcium and phosphorus and consisted of a mixture of 3,632 gm. of ground whole wheat, 136 gm. of wheat gluten, 63 gm. of common salt, and 137 gm. of steamed bone meal, the latter being the same product used in our earlier experiments with cows (6). In addition there were fed 2,588 gm. of cut wheat straw for the amount of grain mixture outlined above. This ration consequently provided about 3 parts of the grain mixture to 2 parts of the straw and was maintained in this proportion as much as possible, although there were times when the ratio varied from these figures. We selected these proportions of ingredients in the ration because they were the same proportions, with the exception of the bone meal, as used in our earlier work with growing heifers (9) and we desired to correlate, if possible, these data with those early observations. Distilled water was allowed *ad libitum* and each animal received in addition 15 cc. of a solution of potassium iodide per day (7 gm. per liter of water).

The animals were confined in a basement room with closed windows, partly shaded, and when not in the metabolism cages they were placed in small pens in the same room. This procedure, as far as we know, prevented any unknown disturbing factor from entering into the experiments. Quantitative collections of all excreta and milk were made with sampling of each for final determinations of calcium and phosphorus.

These experiments were begun in October, 1923, and during the entire summer season from May until October, 1923, all of the animals had been in an outdoor paddock with free access to green grasses and light; consequently there is every probability that they entered the experiment with a reserve store of the accessory factor or factors concerned in this problem. It was planned to bring the animals into negative calcium balance with the above ration and then subject them to ultra-violet light with continued study of their status in reference to calcium and phosphorus equilibrium.

Record of Animal 1.

Wheat Straw Period.—After 3 weeks of preliminary feeding, balances were started October 26, 1923, but since the animal was found to be in positive calcium balance, as can be seen in Table I, she was removed from the cage for 2 weeks. Another trial in November showed that the calcium balance was still positive and, therefore, the animal was allowed to remain in the open pen for 5 weeks during which time she freshened. This animal freshened January 6, 1924, giving birth to two normal young. Removal to the metabolism cage was made as soon after freshening as possible with the result that the animal was found to have a distinctly negative calcium balance. This clearly demonstrates the increased drain upon the animal of both calcium and phosphorus and the factor influencing their assimilation during lactation. Before lactation this animal had maintained a positive calcium equilibrium although unquestionably this could not have been sustained indefinitely. After the drain of milk production was imposed upon the organism the store of the antirachitic factor was no doubt quickly reduced. The number of grams of calcium eliminated in the milk per week was no greater than the number of grams stored per week previously, but instead of coming to an equilibrium at a lower positive balance, a marked negative balance for calcium was found.

TABLE I.
*Record of Calcium Balance and Milk Production of Animal I.**

Date.	Dried feces.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Wheat straw period.								
<i>1923-24</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>cc.</i>
Oct. 26-Nov. 2...	1,096	31.37	1.62		32.39	33.39	+1.00	
Nov. 2-9.....	1,117	35.65	0.13		35.78	33.39	-2.39	
“ 9-16.....	Same ration but outside cage.							
“ 16-23.....	“	“	“	“	“			
“ 23-30.....	1,113	29.82	0.17		29.99	35.77	+5.78	
“ 30-Dec. 7...	1,137	28.99	0.20		29.19	35.77	+6.58	
Dec. 7-14.....	1,090	32.48	0.28		32.76	35.77	+3.01	
“ 14-21.....	Same ration but outside cage.							
“ 21-28.....	“	“	“	“	“			
“ 28-Jan. 4...	“	“	“	“	“			
Jan. 4-11.....	“	“	“	“	“			
“ 11-18.....	“	“	“	“	“			
“ 18-25.....	1,265	39.09	0.22	5.90	45.21	36.19	-9.02	2,695
“ 25-Feb. 1...	1,160	24.47	0.09	5.23	29.79	35.47	+5.68	2,490
Feb. 1-8.....	1,115	34.56	0.06	4.99	39.61	35.51	-4.10	2,325
“ 8-15.....	1,110	33.30	0.17	4.63	38.10	35.97	-2.13	2,205
“ 15-22.....	1,200	36.00	0.14	4.82	40.96	28.87	-12.09	2,190
“ 22-27.....	667	16.00	0.10	2.65	18.75	18.78	+0.03	1,205
Radiated 10 min. per day.								
Feb. 27-Mar. 5...	1,125	34.76	0.16	3.88	38.80	34.24	-4.56	1,765
Mar. 5-12.....	1,090	31.06	0.16	3.38	34.60	33.97	-0.63	1,610
Radiated 20 min. per day.								
Mar. 12-19.....	1,033	29.02	0.15	3.21	32.38	34.24	+1.86	1,605
“ 19-26.....	980	31.55	0.12	2.55	34.22	34.66	+0.44	1,525
“ 26-Apr. 2...	1,020	28.45	0.25	2.81	31.51	34.25	+2.73	1,480
Apr. 2-9.....	1,089	29.62	0.21	2.42	32.23	31.82	-0.41	1,275
“ 9-16.....	1,067	28.38	0.45	2.29	31.12	33.86	+2.74	1,145
Yellow corn addition and low calcium level + radiation.								
Apr. 16-23.....	1,062	25.59	0.30	2.15	28.04	25.06	-2.98	1,075
“ 23-30.....	1,064	24.26	0.27	1.87	26.40	25.98	-0.42	935
“ 30-May 7...	1,054	23.82	0.07	1.68	25.57	24.66	-0.91	855
May 7-14.....	1,100	23.32	0.14	1.71	25.17	24.04	-1.13	855
Yellow corn addition and high calcium level + radiation.								
May 14-21.....	998	22.85	0.29	1.36	24.50	29.59	+5.09	650
“ 21-28.....	962	19.81	0.25	1.17	21.23	24.32	+3.09	585
“ 28-June 4...	885	16.37	0.07	0.86	17.30	20.17	+2.87	390
June 4-11.....	930	15.99	0.17	0.79	16.95	20.08	+3.13	285

* Freshened Jan. 6, 1924.

This situation we have often observed; with a liberal calcium and phosphorus intake with natural foods a mature dry animal passes into negative calcium and phosphorus balances only very slowly. In fact, it may take months to show this condition on

TABLE II.
Record of Phosphorus Balance of Animal I.

Date.	Dried. feces.	P ₂ O ₅ in feces.	P ₂ O ₅ in urine.	P ₂ O ₅ in milk.	Total P ₂ O ₅ excreted.	Total P ₂ O ₅ intake.	Balance per week.
Wheat straw period.							
1924	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Feb. 1-8.....	1,115	46.38	0.16	5.58	52.12	57.67	+5.55
“ 8-15....	1,110	43.95	0.09	5.73	49.77	58.10	+8.33
“ 15-22.....	1,200	47.28	0.18	5.25	52.71	50.84	-5.43
“ 22-27.....	667	20.07	0.03	3.01	23.11	29.20	+6.09
Radiated 10 min. per day.							
Feb. 27-Mar. 5...	1,125	45.67	0.12	4.41	50.20	54.80	+4.60
Mar. 5-12.....	1,090	41.74	0.05	4.02	45.81	54.32	+8.51
Radiated 20 min. per day.							
Mar. 12-19.....	1,033	37.70	0.05	4.00	41.75	54.80	+13.05
“ 19-26.....	980	38.51	0.16	3.81	42.48	54.80	+12.32
“ 26-Apr. 2...	1,020	37.23	0.15	3.70	41.08	54.44	+13.36
Apr. 2-9.....	1,089	37.68	0.29	3.20	41.17	51.98	+10.81
“ 9-16.....	1,067	37.45	0.23	2.86	40.54	52.20	+11.66
Yellow corn addition and low calcium level + radiation.							
Apr. 16-23.....	1,062	34.51	0.17	2.68	37.36	44.14	+6.78
“ 23-30.....	1,064	33.62	0.12	2.34	36.08	46.06	+9.98
“ 30-May 7...	1,054	33.41	0.09	2.13	35.63	42.64	+7.01
May 7-14.....	1,100	32.34	0.12	2.13	34.59	40.51	+5.92
Yellow corn addition and high calcium level + radiation.							
May 14-21.....	998	34.43	0.15	1.62	36.20	43.23	+7.03
“ 21-28.....	962	29.34	0.19	1.46	30.99	35.54	+4.55
“ 28-June 4...	885	23.27	0.23	1.09	24.59	28.16	+3.57
June 4-11.....	930	25.82	0.17	0.77	26.76	28.64	+1.88

what we consider a very severe ration, that is, one low in the antirachitic factor; but as soon as milk production is imposed upon the animal, even with liberal calcium and phosphorus supplies, a negative calcium balance is soon established.

With the liberal phosphorus supply, as furnished by our ration, a negative phosphorus balance was not established coincident with a negative calcium balance. (See Table II.) This condition has been observed before, there being a certain amount of independence in the metabolism of the elements, calcium and phosphorus.

From this period on the animal remained in a distinctly negative calcium balance, with the exception of 1 week, which is in harmony with earlier observations where negative calcium balances were obtained with lactating goats on oat straw plus a grain ration. Although this animal was receiving twice as much calcium oxide per week as animals used in our earlier work, nevertheless, a distinctly negative balance was maintained, indicating that after the store of the antirachitic factor was once depleted the level of calcium intake would have little effect upon calcium equilibrium.

Radiation Period.—The feeding during this period was exactly as in the previous period except that in addition to the ration the animal was radiated 10 minutes per day for the first 2 weeks and thereafter for 20 minutes per day. Radiation was secured by exposure to the rays of a quartz mercury vapor lamp at a distance of 2 feet. The animal remained negative during the 1st week of radiation but in the next week the calcium elimination was distinctly reduced in amount. The significant thing to be observed is that with radiation there was a gradual reduction in the calcium content of the feces, although the amount of calcium ingested in the ration remained practically the same.

The following week the length of exposure to ultra-violet light was increased to 20 minutes per day because we thought that possibly 10 minutes per day was not sufficient. Upon radiation for 20 minutes per day the animal was changed to a positive calcium balance and remained positive for a period of 5 weeks under the influence of the daily radiation with the exception of 1 week when the balance was slightly negative, due to a poor appetite which decreased the calcium intake. That this animal, from a distinctly negative calcium balance, could be brought to a positive calcium balance by exposure to ultra-violet light shows without doubt that ultra-violet light has the same effect in maintaining proper calcium and phosphorus equilibrium in the blood stream of mature animals as it has in young growing animals, and that

under these conditions the experiment indicates its equivalence to the antirachitic factor.

Period of Lower Calcium Intake.—Since this animal had been on a ration deficient in vitamin A as well as the antirachitic factor for such a long period of time, one-third of the wheat meal was replaced by yellow corn which would furnish a liberal supply of vitamin A. The introduction of yellow corn would have no effect on the antirachitic deficiency because it has been shown (6) that liberally milking cows can be kept in a negative calcium balance on rations in which the grain portion contained 60 per cent of yellow corn. In order to determine if a *positive* calcium balance could be maintained by radiation if the calcium intake was lowered, the calcium oxide content of the grain mixture was reduced one-fourth by using less of the steamed bone meal. A *negative* calcium balance of almost 3 gm. was observed in the 1st week after the calcium reduction in the ration was made, due to the sudden lowering in intake and the lag in elimination of calcium. The 3 following weeks on the lower calcium intake showed just slight negative balances, which indicates that at this lower level of calcium intake a definite positive balance could not be maintained with a milking goat, although the ultra-violet light still had a decided effect in reducing the degree of negativity to a minimum.

Period of Higher Calcium Intake.—Because of the fact that we did not establish through radiation with ultra-violet light absolutely positive calcium balances on the *lower calcium intake*, the calcium in the ration was again raised to approximately what it was in the earlier periods of the experiment. At this higher level of calcium intake and with continued daily radiation very decided positive calcium balances were again established. The animal continued to decrease her milk flow until it had receded from an initial volume per week of approximately 2 liters to 400 to 500 cc. Due to the reduction in milk volume the intake of the grain mixture was somewhat reduced and at the same time the calcium intake was gradually being reduced; but with the decreased secretion of calcium into the milk, even with lower levels of calcium intake, distinctly positive calcium balances were established.

Blood Analysis.—The blood from this goat was analyzed for total calcium and inorganic phosphorus before and after radiation. The method used for calcium was that of DeWaard (10) and for inorganic phosphorus the Briggs' (11) modification of the Bell-Doisy method was used. Before radiation the blood contained 9.92 mg. of calcium per 100 cc. of serum and 5.94 mg. of inorganic phosphorus per 100 cc. of serum. When the animal had been radiated for 5 weeks the blood contained 9.43 mg. of calcium per 100 cc. of serum and 7.80 mg. of inorganic phosphorus per 100 cc. of serum. When the experiment was terminated the blood was again taken, and at this time the total calcium in the blood was 9.31 mg. per 100 cc. of serum and 8.33 mg. of inorganic phosphorus per 100 cc. of serum. These results indicate a distinct influence on the inorganic phosphorus content of the blood through radiation,—a fact that has already been observed with growing animals.

Record of Animal 2.

Wheat Straw Period.—This animal was placed on our restricted ration similar to that fed Animal 1 on December 15, 1923. A metabolism trial in February, 1924, showed that she was still in positive calcium balance and consequently was left in the pen, but fed the same ration until after freshening. On March 21, 1924, she gave birth to two young and developed a large milk flow amounting to over 1,000 cc. per day for the 1st few weeks. Just 1 week after freshening the animal was placed in the metabolism cage and the record for the 1st week showed a positive calcium balance, but from then on distinctly negative calcium balances were obtained practically every week. The results secured with this animal parallel closely the results secured with Animal 1. The fact that she was in positive calcium balance for a short time after freshening can be accounted for by the fact that this animal was removed from green pasture later than Animal 1, and therefore had a larger store of the antirachitic factor at the time of freshening, which kept her in positive balance for a longer time than the other animal. Milking, however, rapidly depleted this factor even if the reserve of it was greater than that of Animal 1 in the prefreshening period.

Radiation Period.—After distinctly negative calcium balances were established, this animal was exposed to the radiations from

a quartz mercury vapor lamp for periods of 20 minutes daily. The nature of the ration and the amounts of calcium and phosphorus ingested were similar to the preradiation period, although there were slight reductions in calcium intake due to slight reduction in food consumption. Under the influence of radiation with ultra-violet light positive calcium balances were immediately established amounting to 3 to 4 gm. per week with a reduction

TABLE III.
*Record of Calcium Balance and Milk Production of Animal II.**

Date.	Dried feces.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Wheat straw period.								
1923-24	gm.	gm.	gm.	gm.	gm.	gm.	gm.	cc.
Dec. 15-Feb. 16...	On ration but no record taken.							
Feb. 16-23.....	2,210	48.18	0.24		48.42	50.67	+2.25	
" 23-Mar. 29...	On ration but no record taken.							
Mar. 29-Apr. 5...	2,047	48.72	0.53	12.67	61.92	66.15	+4.23	7,920
Apr. 5-12.....	1,860	47.43	0.24	11.44	59.11	56.65	-2.46	7,290
" 12-19.....	1,805	51.26	0.44	10.78	62.48	52.25	-10.13	5,990
" 19-26.....	1,775	47.20	0.47	8.84	56.51	57.43	+0.92	5,200
" 26-May 3...	1,786	48.04	0.25	6.69	54.99	52.99	-2.00	3,985
May 3-10.....	1,485	36.68	0.35	7.56	44.59	41.34	-3.25	4,200
" 10-17.....	1,689	45.09	0.26	5.94	51.29	50.82	-0.47	3,415
" 17-24.....	1,632	44.39	0.24	5.56	50.19	50.82	+0.62	3,090
Radiated 20 min. daily.								
May 24-31.....	1,540	40.50	0.20	4.62	45.32	48.52	+3.20	2,615
" 31-June 7...	1,572	40.08	0.15	3.62	43.85	48.53	+4.68	1,850
June 7-14.....	1,600	43.20	0.12	2.94	46.26	50.82	+4.56	1,505
" 14-21.....	1,420	38.48	0.12	2.62	41.22	45.51	+4.29	1,380

* Freshened Mar. 21, 1924.

in the fecal calcium. The radiation was continued for 4 weeks and positive calcium balances prevailed throughout the entire period. Records of both calcium and phosphorus balances for this animal are given in Tables III and IV. As in the case of Animal 1 the phosphorus balance had remained positive during the entire period of record, including the preradiation period.

Blood Analysis.—Before radiation the blood was analyzed for inorganic phosphorus and calcium. The inorganic phosphorus

amounted to 5.55 mg. per 100 cc. of serum, while the total calcium amounted to 9.39 mg. 4 weeks after initial radiation the blood was again analyzed with the following results: total calcium 9.59 mg. per 100 cc. of serum; inorganic phosphorus 9.26 mg. per 100 cc. of serum. As in the case of Animal 1 the inorganic phosphorus of the blood had been distinctly increased through radiation. No particular change in the total calcium had occurred.

TABLE IV.
Record of Phosphorus Balance of Animal II.

Date.	Dried feces.	P ₂ O ₅ in feces.	P ₂ O ₅ in urine.	P ₂ O ₅ in milk.	Total P ₂ O ₅ excreted.	Total P ₂ O ₅ intake.	Balance per week.
Wheat straw period.							
1923-24	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dec. 15-Apr. 5...	On ration but no record taken.						
Apr. 5-12.....	1,860	63.43	0.96	17.31	81.70	93.24	+11.54
“ 12-19.....	1,805	68.05	0.68	12.58	81.31	78.68	-2.63
“ 19-26.....	1,775	61.59	1.32	10.92	73.83	86.87	+13.04
“ 26-May 3...	1,786	63.58	1.08	8.37	73.03	80.62	+7.58
May 3-10.....	1,485	46.92	0.96	8.82	56.70	61.69	+4.99
“ 10-17.....	1,689	64.35	1.00	7.17	72.52	76.96	+4.44
“ 17-24.....	1,632	56.79	0.84	6.49	64.12	76.96	+12.84
Radiated 20 min. daily.							
May 24-31.....	1,540	50.82	0.88	5.49	57.19	73.12	+15.93
“ 31-June 7...	1,572	58.16	1.08	3.88	63.21	73.57	+10.36
June 7-14.....	1,600	55.20	1.00	3.16	59.38	74.12	+14.74
“ 14-21.....	1,420	50.13	1.16	2.90	54.19	66.83	+12.64

Record of Animal 3.

Wheat Straw Period.—This animal was a *mature dry* goat and was placed in the metabolism cage October 6, 1923, after 3 weeks of preliminary feeding. Upon determining the calcium balance for 2 weeks it was found that she was in positive balance, and was, therefore, removed from the cage to the open pen. From then on the animal was placed in the cage at successive intervals and records were made as shown in Tables V and VI. A distinctly positive calcium balance was obtained every week a determination was made with the exception of 2 weeks when her appetite was poor which resulted in decreased calcium intake. These results were somewhat surprising because we (5) had been able to

bring goats into negative calcium balance in a few weeks on an apparently similar ration, *but which contained less calcium*. This present ration which contained a more liberal amount of calcium was able to maintain this animal in positive calcium balance for a period of 29 weeks or over 7 months. The difference in the two experiments cannot be due to a variation in the amount of antirachitic factor retained from the summer period when the animals

TABLE V.
Record of Calcium Balance of Animal III.

Date.	Dried feces.	CaO in feces.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.
Wheat straw period.						
<i>1923-24</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Oct. 26-Nov. 2...	975	30.73	1.78	32.51	33.39	+0.88
Nov. 30-Dec. 7...	842	23.49	0.19	23.68	25.55	+1.87
Jan. 18-25.....	1,065	33.44	0.26	33.70	36.19	+2.49
Feb. 29-Mar. 7...	1,155	24.25	0.19	24.44	26.02	+1.58
Mar. 14-21.....	950	27.55	0.04	27.59	29.87	+2.28
Apr. 23-30.....	1,165	33.32	0.12	33.44	33.88	+0.44
“ 30-May 7...	1,142	35.51	0.12	35.63	33.18	-2.45
May 7-June 11...	On ration but no record taken.					
June 11-18.....	1,000	29.30	0.16	29.46	33.23	+3.77
“ 18-25.....	935	26.08	0.13	26.21	24.58	-1.63
Lower calcium level.						
June 25-July 2...	995	22.19	0.10	22.29	21.28	-1.01
July 2-9.....	1,065	22.47	0.20	22.67	23.59	+0.92
“ 9-16.....	1,015	22.02	0.17	22.19	22.78	+0.59
Radiated 20 min. daily.						
July 16-23.....	1,015	17.86	0.10	17.96	20.35	+2.39
“ 23-30.....	930	19.08	0.17	19.23	22.25	+3.02
“ 30-Aug. 6...	860	15.31	0.13	15.44	21.07	+5.63

ranged freely upon green material. *It must be due to a difference in the level of calcium intake*. If the calcium and phosphorus intakes are high or at least liberal in quantity less of the antirachitic factor is necessary for experimental demonstration of storage, and, of course, with a *non-milking* animal the reserve of this factor accumulated during the summer period is exhausted at a slower rate and therefore can aid in maintaining the animal in positive

calcium balances for a much longer time. This is a very important fact to be kept in mind in estimating the antirachitic property of food materials, because it is apparent that liberal and possibly optimum levels of calcium and phosphorus intake with a small supply of the antirachitic factor will accomplish what a more liberal supply of the antirachitic factor and lower levels of calcium and phosphorus can accomplish. Although this animal had been on our ration since October 26, 1923, distinct and continuous negative balances did not appear even as late as May and June,

TABLE VI.
Record of Phosphorus Balance of Animal III.

Date.	Dried feces.	P ₂ O ₅ in feces.	P ₂ O ₅ in urine.	Total P ₂ O ₅ excreted.	Total P ₂ O ₅ intake.	Balance per week.
Wheat straw period.						
1923-24	gm.	gm.	gm.	gm.	gm.	gm.
Oct. 26-June 18...	On ration but no record taken.					
June 18-25.....	935	32.87	0.17	33.04	38.14	+5.10
Lower calcium level.						
June 25-July 2...	995	34.12	0.12	34.34	37.32	+2.98
July 2-9.....	1,065	34.72	0.11	34.83	42.28	+7.45
" 9-16.....	1,015	30.85	0.21	31.06	40.54	+9.48
Radiated 20 min. daily.						
July 16-23.....	1,015	30.85	0.18	31.03	35.32	+4.29
" 23-30.....	930	31.80	0.14	31.94	39.72	+7.78
" 30-Aug. 6...	860	29.32	0.15	29.47	36.88	+7.41

1924. Consequently the calcium content of the ration was reduced from a weekly intake of over 30 gm. of calcium oxide to one of approximately 20 gm. of calcium oxide. On this lower level of calcium intake an approach to calcium equilibrium was established, but not a continuously negative balance. No doubt a still lower calcium intake would have been necessary in order to put the animal into a continuously negative calcium balance. Although we would have preferred to establish such a condition before exposing the animal to ultra-violet light, yet we proceeded to radiate for 20 minutes daily with the records showing a status approaching calcium equilibrium. The results secured justified our experimental procedure. As shown in Table V there was

a rise in the amount of calcium stored following the exposure to ultra-violet light. At the end of 3 weeks of exposure there was a positive balance of 5.63 gm. of calcium oxide per week.

Even of greater significance than the storage of calcium was the marked fall in the calcium excreted in the feces after radiation was begun. This declined from 22 gm. per week to about 15 gm. per week on an equal intake for both periods. These results harmonize with those secured with the *milking* animals and show the important part played by light in favorably influencing the assimilation and storage of calcium and phosphorus in mature *dry* animals.

In Table VI is shown the record of the phosphorus balance for this animal. At no time was there a negative phosphorus balance, the provision of this element being fairly liberal throughout the entire period of observation.

Previous to radiation a blood analysis showed 5.10 mg. of inorganic phosphorus per 100 cc. of serum and 9.52 mg. of calcium. After radiation had been in progress for 3 weeks analysis of the blood was again made at which time there were 6.60 mg. of inorganic phosphorus in 100 cc. of serum and 9.64 mg. of calcium per 100 cc. of serum. As in the case of the milking animals there was an appreciable rise in the amount of inorganic phosphorus in the blood after exposure to ultra-violet light.

SUMMARY.

1. This paper presents data showing that ultra-violet light can influence the storage of calcium and phosphorus and the equilibrium of these elements in the blood stream of mature animals in a way similar to its effects upon growing animals.

2. Two mature, *lactating* goats and one mature, *dry* goat were brought into distinctly negative calcium balances, or calcium equilibrium, on a ration deficient in the antirachitic factor; upon exposure for 20 minutes daily to the emanations from a quartz mercury vapor lamp negative calcium balances were changed to distinctly positive balances. The inorganic phosphorus of the blood was also appreciably increased through radiation.

3. On similar calcium and phosphorus levels of intake and in the presence of low amounts of the antirachitic factor, mammary secretion brings on rapidly a negative calcium balance as compared to a *non-lactating animal*.

4. These results have a direct bearing upon the maintenance of calcium and phosphorus equilibrium in the dairy cow through the influence of sunlight. Probably sunlight is of more practical importance in these relations to calcium and phosphorus assimilation in cattle than is green plant tissue. This problem will be studied further.

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A METHOD FOR THE DETERMINATION OF LIPOID PHOSPHORUS IN BLOOD AND PLASMA.

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When work was undertaken in this laboratory on the lipid phosphorus of blood plasma it was hoped that the admirable method of phosphorus determination devised by Bell and Doisy,¹ which had been found so useful for inorganic phosphates, could be utilized for this purpose. But the digestion procedure described by them under the subtitle "Total acid-soluble phosphorus" failed in our hands to give satisfactory results with potassium acid phosphate solutions of known phosphorus content. Since it appeared probable that the inaccuracies arose from the variable acidity of the digestion product, the writer resorted to the procedure of Randles and Knudson,² which provides somewhat more precise control of this factor. The results were then as a rule quite satisfactory. But occasional errors, usually negative, rarely positive, on known phosphate solutions, have shaken the writer's confidence in this procedure and led him to devise a method not quite so simple but which is believed to be more reliable and which it is the purpose of the present paper to describe.

The chief difficulty in carrying out the procedure of Randles and Knudson has been encountered in the control of the digestion process. If the heating is not vigorous enough to drive off all the nitric acid, full color development in subsequent stages is prevented. On the other hand, too vigorous heating may also produce errors, usually negative, rarely positive. The negative errors are attributable to local overheating, since the small volume of fluid present at the end of digestion is insufficient to

¹ Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

² Randles, F. S., and Knudson, A., *J. Biol. Chem.*, 1922, liii, 53.

cover completely the bottom of the test-tube. The loss may be by volatilization,³ by conversion to pyro- or metaphosphoric acid,¹ or by formation of silicophosphoric acid.⁴ If, by good fortune, no orthophosphoric acid is lost under these circumstances, the driving off of sulfuric acid by vigorous digestion may reduce the acidity enough to permit a greater color yield than in the standard test-tube, hence a positive, rather than a negative, error.

It is perhaps justifiable, therefore, to express the suspicion that the approximately correct value usually obtained by a vigorous digestion, using the Randles and Knudson procedure, is dependent on a balancing of these two tendencies to error—that the greater color yield permitted by a reduction in the amount of sulfuric acid compensates for the loss of a little orthophosphoric acid. The practical difficulty comes in knowing in a given digestion just when this balance is reached and there is no reliable criterion on this point. Comparison with the volume in a similar test-tube containing 6 drops of sulfuric acid and 3 beads, as proposed by Randles and Knudson, has not been, for the writer's eye, sufficiently precise.

Some idea of the frequency and relative importance of the errors encountered may be gained from an inspection of the following series of determinations on portions of acid potassium phosphate solutions containing 0.04 mg. of P. The standard, prepared from an equal volume of the same solution, was in each case set at 20.0 mm. The colorimeter readings were, in mm.: 20.4, 20.2, 19.7, 23.0, 20.4, 22.8, 23.2, 21.5, 24.0, 19.1, 20.0, 19.9, 18.0, 19.8, 23.0, 20.6.

It has been found possible to overcome the difficulties of the digestion process, without giving up the use of nitric acid. The errors due to local overheating have been avoided by the use

³ Baumann, E. J., *J. Biol. Chem.*, 1924, lix, 667.

⁴ If a very vigorous Randles and Knudson digestion be carried out in a test-tube and the tube rinsed about six times with distilled water, the presence of a little phosphoric acid can usually be demonstrated in the tube by digestion with sulfuric and nitric acids and subsequent color development as described in the following pages, indicating that a little of the phosphoric acid had united with the glass. This may be observed even when the full theoretical color yield has been obtained from the combined rinsings.

of enough sulfuric acid to fill the hemispherical end of the test-tube (1 cc. of specific gravity 1.84), and the traces of nitric acid remaining after digestion are eliminated by sulfur dioxide liberated from sodium sulfite. The use of so much acid also reduces to relative insignificance the amount of sulfuric acid volatilized during digestion.

The intense acidity of the digestion product has necessitated a departure from the process of color development originally described by Bell and Doisy¹ or modified by Briggs.⁵ With this degree of acidity, 10 minutes in boiling water after the addition of acid molybdate, sodium sulfite, and hydroquinone produce a clear blue color, whose intensity, after cooling, is proportional to the quantity of orthophosphoric acid even when the difference exceeds 33 per cent. A blank determination gives only a faint yellow color.⁶ The blue color increases slowly for about 24 hours, and does not fade out completely for several weeks. There is no difficulty in comparing several unknowns with the same portion of standard in the colorimeter, which fact eliminates one of the worrisome features of the Bell-Doisy technique. If the blue solution is diluted with distilled water, the intensity of color varies in inverse proportion to the dilution. This enables one to adjust unexpectedly deep colors nearly to the color of the standard, thus enabling one to carry through an occasional determination which might otherwise have to be discarded.

Reagents.

All solutions must, of course, be made up with phosphate-free water. If a blank determination gives a blue color, the contaminated reagent must be detected and eliminated.

1. *Molybdic Acid*.—5 per cent ammonium molybdate in 2 N sulfuric acid. This keeps indefinitely and deposits less sediment than the Bell-Doisy solution which is made with 1 N acid.

2. *Hydroquinone*.—1 per cent. This keeps several weeks if preserved with 2 to 4 drops of concentrated sulfuric acid per 100 cc., and tightly stoppered.

⁵ Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

⁶ On this account it is inadvisable to attempt a determination on less than 0.02 mg. of P since the resulting blue color has a greenish tinge because of the *relatively* large admixture of yellow.

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3. *Sodium Sulfit*.—20 per cent of the anhydrous salt. In tightly stoppered stock bottles it may keep for many months, but it is probably wiser to prepare only a few weeks' supply at a time.

4. *Standard*.—Finely ground monopotassium phosphate is dried over sulfuric acid for a week. 4.390 gm. of this dry salt and 20 cc. of concentrated sulfuric acid are dissolved in distilled water and the volume is made to 1 liter. This keeps indefinitely and is more neatly handled with a pipette than when preserved with chloroform. A suitable standard for blood is obtained by diluting 1 cc. of this stock standard to 100 cc. 5 cc. contain 0.05 mg. of phosphorus. If it is desired to keep this dilute standard more than a week it should be more strongly acidified with 3 drops of concentrated sulfuric acid.

5. *Alcohol-Ether Mixture*.—This consists of 3 volumes of redistilled ethyl alcohol and 1 volume of redistilled ether.

Procedure.

Into a glass-stoppered 100 cc. volumetric flask pour about 75 cc. of the alcohol-ether mixture, whirl the flask a few times, and as the contents rotate add, in a fine stream from a pipette, 5 cc. of whole blood or plasma oxalated to prevent clotting. Immerse in hot water, continue the rotation gently until the mixture just boils, then cool with running water, add alcohol-ether mixture to the mark, mix, and filter. Cover the funnel with a watch-glass during filtration and stopper the bottle containing the filtrate as soon as it is collected. When economy of material is required, 1 cc. can be extracted in a 50 cc. flask. Then, of course, two and a half times as much filtrate will be needed for a determination.

Pipette 10 cc. of the "lipoid" extract into a Pyrex test-tube, 25 × 200 mm., add a small piece of quartz, and evaporate to dryness in a hot water bath, taking care to prevent boiling over. Add 1 cc. each of concentrated sulfuric and concentrated nitric acids,⁷ and digest over the naked flame of a micro burner. Continue digestion for 10 minutes after white fumes appear, heating just rapidly enough to keep the tube nearly filled with fumes.

If a thin wisp of fumes escapes from the test-tube it does not upset the determination, but if the heating is so vigorous as to drive off dense fumes phosphoric acid may be lost by volatiliza-

⁷ The acids may be added from dropping bottles which have been roughly calibrated by counting the number of drops required to fill 1 cc. in a small graduated cylinder.

tion. With the gas supply in this laboratory, satisfactory digestion is obtained by placing the bottom of the tube about 1 cm. above the luminous tip of a $1\frac{1}{2}$ cm. flame, protected from air currents by a glass shield.

Let cool for 3 minutes, add cautiously 2 cc. of 20 per cent sodium sulfite solution and another small piece of quartz, mix, and heat at the same rate as before for 2 minutes. The heating should not be carried beyond the time when white fumes appear, which usually occurs in about 4 minutes, since the higher temperature of the salt-acid digestion may convert some orthophosphoric into pyrophosphoric acid.

After cooling a minute or more, add 5 cc. of distilled water and place the tube in cold water. In a similar tube put 1 cc. of concentrated sulfuric acid, 2 cc. of 20 per cent sodium sulfite, and 5 cc. of the blood-standard phosphate solution (0.05 mg. of P) and place in cold water. When cool, add to each 2 cc. of acid molybdate solution, 2 cc. of sodium sulfite solution and 1 cc. of hydroquinone solution, shaking after each addition. Then transfer each to a test-tube, 16×170 mm., marked at 15, 20, and 25 cc., and add water to the 15 cc. mark. Stopper with clean thumb and invert twice. Set in vigorously boiling water bath for 10 minutes.⁸ Cool at the tap. Add water to the standard up to the 25 cc. mark and mix. The unknown may be made up to 15, 20, or 25 cc. with water, whichever gives a color approximately like the standard. Having assured oneself that the standard reads 20/20 against itself, the unknown is matched against the standard set at 20.0 mm. in a Duboseq colorimeter, and the lipid phosphorus content of the blood or plasma calculated in the manner described below. A series of four or five unknowns can be carried through together and read successively against the same portion of standard.

Calculation.—Since the amount of extract taken represents 0.5 cc. of blood (or plasma), the lipid phosphorus content of 100 cc. is given by the formula $\frac{20}{R} \times \frac{V}{25} \times 0.05 \times 200$ or $\frac{V}{R} \times 8$, in which R represents the colorimeter reading and V the volume (in cc.) to which the unknown was made up before reading.

⁸ In a recent article (Briggs, A. P., *J. Biol. Chem.*, 1924, lix, 255) Briggs describes a method for color development in which the addition of sulfite is preceded by a half hour's heating. However, in the procedure described above, more color is obtained by adding the sulfite before heating, and the proportionality between color and phosphorus is maintained.

Accuracy and Reliability.

The procedure as detailed has given very satisfactory results on known solutions of inorganic orthophosphates. The following results were obtained in a consecutive series: 0.02 mg. of P gave 0.0189, 0.0191, and 0.020; 0.036 mg. of P gave 0.0356 and 0.0362; 0.04 mg. of P gave 0.0402, 0.0398, 0.0388, 0.0410, 0.0408, 0.0385, 0.0375, 0.0375, 0.0389, and 0.040; 0.06 mg. of P gave 0.0588, 0.060, 0.0595, 0.0602, 0.0588, 0.0606, and 0.0570; and 0.08 mg. of P gave 0.0784.

It seemed wise to test, also, if the procedure were adequate for the digestion of organic phosphorus compounds. For this purpose a sample of highly purified lecithin, and a sample of rather crude sodium nucleate, both prepared from adrenal glands, were used. The method gave 0.0720 and 0.0709 mg. of P from 1.872 mg. of lecithin (3.85 and 3.79 per cent compared with 3.87 per cent by the macro method), and 0.0348 and 0.0345 mg. of P from 0.447 mg. of nucleate (7.8 and 7.7 per cent compared with 7.6 per cent by the macro method).

The reliability of the whole procedure was also tested by determinations on plasma whose lipoid phosphorus content had been enriched by the addition of aqueous emulsions of lecithin, prepared by adding measured quantities of an alcohol-ether solution of lecithin to water and heating to drive off the organic solvents. In this way the added lecithin mixes very thoroughly with the plasma. Thus to each of four 5 cc. portions of a given plasma, which was found to contain 7.55 mg. of lipoid P per 100 cc., were added 4.69 mg. of lecithin, and the whole of each mixture was used in preparing the alcohol-ether extracts in 100 cc. flasks. Analysis of the filtrates should give $7.55 + 20 \times 0.0387 \times 4.69$ or 11.18 mg. of lipoid P per 100 cc. of plasma. The results were in fact 11.2, 11.4, 11.1, and 11.2. Similarly, another sample of plasma containing 8.95 mg. of P per 100 cc., when enriched to 13.2 gave 13.2 and 13.1.

As a further measure of reliability twelve determinations were carried out on the same plasma,—a sufficient quantity being obtained by pooling several specimens. Expressed as mg. of lipoid P per 100 cc. of plasma, the results were: 9.4, 9.5, 9.5, 9.2, 9.4, 9.4, 9.1, 8.9, 9.2, 9.3, 8.9, 9.2.

INFLUENCE OF GLUCOSE AND FRUCTOSE ON THE RATE OF HYDROLYSIS OF SUCROSE BY INVERTASE FROM HONEY.*

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When the rate of hydrolysis of sucrose, by invertase from honey, is plotted against time, the graph obtained was found, by Nelson and Cohn (1), to indicate an acceleration at the beginning of the reaction. The effect is represented by the first part of Curve *C* in Fig. 1. This increase in the rate of hydrolysis is rather unusual since invertase action, when the invertase is obtained from bottom yeast, does not show this effect.

The present paper is, therefore, an account of experiments carried out with the purpose of making a further study of this effect. The first cause which suggested itself is the influence of invert sugar on the rate of hydrolysis of the sucrose. Consequently, several hydrolyses have been run, using sucrose solutions containing added glucose and fructose.

The method of procedure employed was to make up solutions containing the desired concentrations of sucrose, added hexose, and buffer, usually sodium citrate and hydrochloric acid, so as to furnish the required hydrogen ion concentration. The hydrolyses were run in a thermostat and samples taken at definite time intervals during the course of the reaction. The samples were treated with definite quantities of sodium carbonate solution to interrupt the enzyme action and to bring about mutarotation of the reducing sugars present. The degree of rotation of the samples was determined with mercury light. The invertase EL was prepared from honey according to the method described by Nelson and Cohn (1).

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Effect of Glucose on the Rate of Hydrolysis of Sucrose.

The results recorded in Table I represent hydrolyses, by invertase from honey, of 10 per cent sucrose solution, containing from 0.1 to 5.0 per cent of added mutarotated glucose. In order to have the glucose in the mutarotated form, pure α -glucose was dissolved in water and the solution allowed to stand overnight before being added to the sucrose solution. The hydrolyses were all run at a hydrogen ion concentration of pH 5.76 which has been shown by Nelson and Cohn (1) to be the optimum hydrogen

TABLE I.

Conditions:

pH: 5.76

Temperature: 25°C.

10 per cent invertase %EL.

10 per cent sucrose.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5 cc. Na_2CO_3 .

Varying amounts of mutarotated glucose.

Mutarotated glucose.	Change in rotation at the following time intervals, in min., during the course of the hydrolysis.						
	20	40	60	90	120	195	300
<i>per cent</i>							
0.0	2.02	4.58	7.10	10.33	13.01	17.50	21.46
0.1	2.80	5.56	8.05		13.57	17.89	21.67
0.3	3.28	6.05	8.44		13.73	17.96	21.79
0.4	3.29	6.09	8.39	11.28	13.60	17.78	21.60
0.5	3.38	6.08	8.35	11.17	13.42	17.59	21.40
0.6	3.46	6.17	8.42	11.12	13.45	17.41	21.27
1.0	3.05	5.56	7.76	10.39	12.51	16.62	20.37
2.0	2.38	4.51	6.30	8.56	10.56	14.38	18.14
5.0	1.20	2.44	3.45	4.90	6.18	9.01	12.33

ion concentration for honey invertase. In each hydrolysis, the same conditions, with the exception of the amount of glucose added, were maintained throughout, so the results obtained are comparable. When the concentration of glucose was 2.0 per cent and under, an acceleration of the rate of hydrolysis was obtained. Thus, by comparing the values for change in rotation for a definite length of time, as given in Table I, it will be noticed that the values are larger when 0.1 to 2.0 per cent mutarotated glucose had been added than when no glucose was introduced into the hydrolyzing sucrose solution. It will be seen also that, as the time interval of hydrolysis increases, the

increase in value of the change of rotation becomes less and less, especially for the higher concentrations of added glucose. When the glucose concentration was 5.0 per cent the activity of the enzyme was materially lessened; or in other words, the glucose, beyond this concentration, acted as a retardant. Maximum activity was obtained when the glucose was present in concentrations of 0.5 to 0.6 per cent. It can be seen, therefore, that

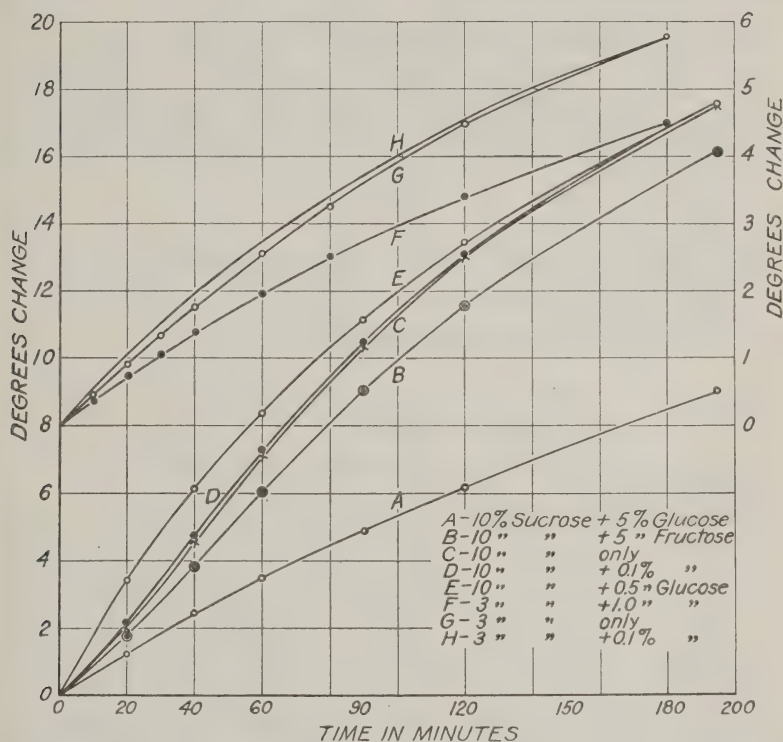


FIG. 1.

the rate of hydrolysis of sucrose, in the presence of invertase extracted from honey, is affected in two ways by the addition of mutarotated glucose depending on the amounts introduced in the hydrolysis mixture.

In addition to the increase and decrease in the activity of the enzyme, the presence of the added glucose, irrespective of amounts, serves to eliminate the initial increase in rate of inversion, ob-

served by Nelson and Cohn, which seems to be characteristic of all hydrolyses of 10 per cent sucrose solutions in the presence of honey invertase. Curve *C*, Fig. 1, representing an ordinary hydrolysis, shows the initial rise. On the other hand, Curves *A* and *E* which represent hydrolyses of 10 per cent sucrose solutions, with 5.0 and 0.5 per cent of added glucose, respectively, show no such initial increase.

Effect of Fructose on the Rate of Hydrolysis of Sucrose.

The next set of experiments is analogous to the first, with the only exception that mutarotated fructose, instead of glucose, was added to the hydrolysis mixture. All other conditions were

TABLE II.

Conditions:

pH: 5.76

Temperature: 25°C.

10 per cent sucrose.

Varying amounts of mutarotated fructose.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5 cc. Na_2CO_3 .

10 per cent invertase %EL.

Mutarotated fructose.	Change in rotation at the following time intervals, in min., during the course of the hydrolysis.						
	20	40	60	90	120	195	300
<i>per cent</i>							
0.0	2.02	4.58	7.10	10.33	13.01	17.50	21.46
0.1	2.12	4.70	7.27	10.48	13.06	17.59	21.58
0.2	2.10	4.67	7.20	10.41	12.99	17.52	21.54
0.5	2.01	4.48	7.01	10.20	12.82	17.36	21.30
1.0	1.98	4.43	6.92	10.10	12.70	17.21	21.14
5.0	1.73	3.84	6.01	9.04	11.54	16.12	20.08

retained so that the results obtained would be comparable to those of glucose. The amounts of fructose added varied from 0.1 to 5.0 per cent. The experimental data are recorded in Table II. Again we observe that fructose, like glucose, causes both retardation and acceleration, depending on the quantities added. But the fructose effect is considerably less in magnitude than that produced by glucose. Thus it will be observed, when the change in rotation number in the 20 minute columns of Tables I and II are compared, that the fructose values in Table II are always less than those for glucose given in Table I. Further-

more it will be noted that maximum acceleration in the presence of fructose is observed when 0.1 per cent is added, while in the case of glucose the maximum is reached in the presence of 0.5 per cent. This difference is not due to the retarding action of fructose, since glucose is a better retardant, as shown by a comparison of Curves *A* and *B* in the figure. Hence, the relative effects obtained by the addition of fructose and glucose appear not to be the same, either in magnitude or quality. When fructose is present in very small amounts, 0.1 and 0.2 per cent, the activity of the enzyme is slightly increased. With a concentration of 0.5 per cent there is practically no effect. If the amounts of fructose are increased we begin to get retardation. Curves *B* and *D* represent hydrolyses of 10 per cent sucrose solutions in the presence of 5.0 and 0.1 per cent fructose, respectively. An examination of the two curves brings out the fact that the accelerating influence of the fructose is not large enough to eliminate the initial rise in the hydrolysis curve as does glucose. This can be clearly seen by comparing the shape of Curves *B* and *D* with Curves *A* and *E*.

Variation of the Sucrose Concentration.

It is a well known fact that the rate of hydrolysis, when invertase from bottom yeast is used, varies with different sucrose concentrations (Michaelis and Menten (2) and Nelson and Bloomfield (3)). Nelson and Cohn (1) showed this to be true also for honey invertase. They also showed that the increase in the initial rate of inversion, noticed in the case of 10 per cent sucrose solution (see Curve *C*), does not occur in more dilute sucrose solutions (3 per cent or less¹). The results obtained, in the present study, with 10 per cent sucrose solutions and varying amounts of added fructose and glucose, indicate that there might be different degrees of acceleration of the rate of hydrolysis when different concentrations of sucrose and the hexoses are used. To this purpose, a third series of experiments was carried out, using a dilute sucrose solution (3 per cent) and varying amounts of mutarotated glucose. This particular sucrose concentration was chosen, because of the above mentioned results

¹ See Nelson and Cohn (1), Figs. 8 and 9.

obtained by Nelson and Cohn. All other conditions were maintained the same as in the previous experiments. The results obtained are recorded in Table III and shown graphically by Curves *F*, *G*, and *H*. It will be seen from the shapes of Curves *F* and *H* that there is no initial increase in the rate of inversion. Furthermore, it will seem, in comparing the positions of Curves *F* and *H* with that of *G* that, in the presence of lower concentrations of sucrose, 0.1 per cent of added glucose causes an increase in the rate of hydrolysis, while 1.0 per cent causes a retardation. Comparing this with the results obtained when

TABLE III.

Conditions:

pH: 5.81

Temperature: 25°C.

3 per cent sucrose.

Varying amounts of mutarotated glucose.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5 cc. Na₂CO₃.

10 per cent invertase %EL 2.*

Mutarotated glucose.	Change in rotation at the following time intervals, in min., during the course of the hydrolysis.							
	10	20	30	40	60	80	120	180
<i>per cent</i>								
0.0	0.45	0.90	1.33	1.74	2.57	3.25	4.48	5.78
0.1	0.58	1.06	1.54	1.96	2.73	3.42	4.54	5.75
0.2	0.55	1.07	1.52	1.93	2.71	3.38	4.48	5.71
0.25	0.54	1.06	1.53	1.93	2.69	3.38	4.48	5.70
0.5	0.50	0.94	1.36	1.76	2.46	3.13	4.16	5.36
1.0	0.38	0.73	1.04	1.36	1.96	2.50	3.41	4.47

*Invertase %EL 2 is the same as %EL with an equal volume of water added.

10 per cent sucrose solution was used, shows that for the dilute sucrose solution, 1.0 per cent glucose acts as a retardant (Table III) while for the more concentrated sucrose solution it behaves as an accelerator. Therefore it is quite evident that the concentration of sucrose is a factor in determining whether the added glucose functions as a retardant or accelerator.

Relative Effects of Mutameric Forms of Glucose and Fructose.

In a sucrose hydrolysis by invertase the reaction occurs in solutions of such low acidity, hydrogen ion concentration, pH 3.5 to 5.0, that mutarotation of the invert sugar formed is rela-

TABLE IV.

Retardant Effects of Mutarotated and α -Glucose.

Conditions:

pH: 5.76

Temperature: 25°C.

10 per cent sucrose.

Glucose: 5 per cent.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5 cc. Na_2CO_3 .10 per cent invertase $\% \text{EL}$.

α -Glucose.			Mutarotated glucose.		
Time.	Reading.	Difference.	Time.	Reading.	Difference.
0	36.20		0	36.20	
10	35.52	0.68	10	35.58	0.62
20	34.86	1.34	20	35.00	1.20
30	34.20	2.00	30	34.36	1.84
40	33.58	2.62	40	33.76	2.44
60	32.54	3.66	60	32.90	3.30

The hydrolysis was started 9 min. after the addition of the α -glucose.

TABLE V.

Accelerating Effects of α - and β -Glucose.

Conditions:

pH: 5.76

Temperature: 25°C.

10 per cent sucrose.

Glucose: 0.5 per cent.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5cc. Na_2CO_3 .10 per cent invertase $\% \text{EL}$.

α -Glucose.			Mutarotated glucose.		β -Glucose.	
Time.	Reading.	Difference.	Reading.	Difference.	Reading.	Difference.
0	26.92		26.91		26.92	
10	25.23	1.69	25.12	1.79	25.09	1.83
20	23.71	3.21	23.52	3.39	23.49	3.43
30	22.29	4.63	22.08	4.83	22.00	4.92
40	20.99	5.93	20.80	6.11	20.71	6.21
60	18.66	8.26	18.53	8.38	18.41	8.51

The hydrolyses were started 6 min. after the addition of the α - and β -glucose.

tively slow. For this reason a considerable portion of invert sugar is present in the hydrolyzing solution in the mutameric form in which it was liberated from the sucrose molecule. Therefore, it was deemed advisable to study also the influence of α - and β -glucose and β -fructose.

It was found (Table IV) that α -glucose retards less than the mutarotated form, which is an equilibrium mixture of the two isomers. This would indicate that the β form has the greater retardant power. Next, a comparison of the relative accelerating power of the two isomers was carried out. The results obtained (Table V) show that α -glucose accelerates less than either the mutarotated or β forms, and that the β -isomer has the greatest accelerating power. Similar experiments were carried out with

TABLE VI.

Retardant Effects of Mutarotated and β -Fructose.

Conditions:

pH: 5.76

Temperature: 25°C.

10 per cent sucrose.

Fructose: 5 per cent.

Polariscope tube: 400 mm.

Samples: 25 cc. + 5 cc. Na_2CO_3 .

10 per cent invertase %EL.

β -Fructose.			Mutarotated fructose.		
Time.	Reading.	Difference.	Time.	Reading.	Difference.
0	8.20		0	8.20	
10	7.45	0.75	10	7.46	0.74
20	6.47	1.73	20	6.47	1.73
30	5.45	2.75	30	5.44	2.76
40	4.37	3.83	40	4.36	3.84
60	2.17	6.03	60	2.19	6.01

The hydrolysis was started 8 min. after the addition of the β -fructose.

fructose. In this case, however, only the retardant effect was considered, since fructose exhibits relatively slight accelerating power. Since the α form has not as yet been obtained in pure crystalline form, the comparison had to be limited to the mutarotated and β forms. It was found (Table VI) that the two forms retarded at the same rate. This would indicate that retardation, in the case of fructose, is independent of mutarotation.

SUMMARY.

1. Mutarotated glucose, besides retarding the action of a preparation of invertase, obtained from honey, when present in higher concentrations, also has an accelerating influence when present in lower concentrations.

2. α -Glucose retards or accelerates, depending upon its concentration, less than the β or mutarotated forms.

3. Mutarotated and β -fructose do not retard nor accelerate to the same extent that the glucose does.

4. There is little or no difference between the relative effects of the various forms of fructose.

5. The presence of added glucose tends to eliminate the characteristic initial increase in the rate of hydrolysis of a 10 per cent sucrose solution by invertase from honey.

6. There appears to be a relationship between the relative concentrations of glucose and sucrose in respect to the extent of the influence of the hexose on the rate of the hydrolysis.

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ON THE PASSAGE OF BORIC ACID THROUGH THE SKIN BY OSMOSIS.

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INTRODUCTION.

Some years ago Dr. Edward H. Ochsner and I made a study of the action of boric acid when patients were treated for blood poisoning by means of compresses of boric acid solutions. The essential results were reported by Dr. Ochsner in a paper on "The treatment of septic infections of the extremities."¹ In that investigation it was found that when aqueous solutions of boric acid, to which alcohol either had or had not been added, are applied to the skin of persons suffering from blood poisoning, the boric acid actually passes through the unbroken skin and makes its appearance in the urine in estimable quantities. The treatment consisted of applying a saturated aqueous solution of boric acid in the form of a large wet dressing to the affected part. This dressing was kept at body temperature, or what was more commonly done, 1 volume of 95 per cent alcohol was added to 3 volumes of the saturated aqueous solution of boric acid. The presence of the alcohol prevents the cold, clammy feeling which a large wet aqueous dressing causes when it is not kept warm. To keep such a purely aqueous dressing at body temperature, or a little above, for hours at a time would require constant attention on the part of the nurse. Various strengths of boric acid were tried in this research, but the best results were obtained when a saturated solution was used. This is what one would have expected since boric acid is but sparingly soluble, the saturated solution at ordinary temperatures containing only about 4 per cent. In all of the cases treated, the amount of

¹ Ochsner, E. H., *Med. Herald*, 1911, xxx, 33.

boric acid found in the urine was small. It very seldom was as much as 1 per cent, generally being only a few hundredths of a per cent, or at best 0.1 or 0.2 of a per cent. The boric acid was commonly found in the urine after an hour's treatment. The length of time required for the boric acid to make its appearance in the urine in detectable quantity was not a special object of the investigation, for that would naturally depend upon the area treated, the care used in cleansing the skin before putting on the compress, and keeping the latter warm and in perfect contact with the skin. The treatments were continued for 48 hours and the urine was examined from time to time for 46 hours after removing the compress. It was thus definitely established that boric acid is actually absorbed by the skin, and that it is eliminated by the kidneys, traces of boric acid being still found in the urine 46 hours after the topical application had ceased.

These investigations on the use of boric acid in the treatment of infections interested me in the experimental study of osmosis, and the results of the osmotic studies carried on were published in a series of papers from this laboratory, the basal one being entitled, "On the nature of the process of osmosis and osmotic pressure with observations concerning dialysis."² In that paper it has been conclusively demonstrated by experiment that *"whether osmosis will take place in a given case or not depends upon the specific nature of the septum and the liquids that bathe it; and if osmosis does occur, these factors also determine the direction of the main osmotic current and the magnitude of the osmotic pressure developed."* All osmotic experiments made since the publication of that basal article have served to confirm this conclusion, which is also substantiated by the work of earlier investigators. To be sure, these osmotic experiments were performed with lifeless membranes, that is to say with dead animal or plant membranes on the one hand, or with inanimate artificial septa on the other.

In the course of my experimental osmotic work I found that not only boric acid but also urea, cane-sugar, sodium chloride, potassium chloride, lithium chloride, rubidium chloride, cesium chloride, sodium sulfate, and borax all pass through animal membranes such as the pericardium, urinary bladder, gall cyst,

² Kahlenberg, L., *J. Phys. Chem.*, 1906, x, 141.

stomach, intestine, aorta, and diaphragm of the hog, cow, or sheep; also through dead human skin. The latter was obtained from the dissecting room of the anatomical department and was taken from the outer wall of the abdomen. Osmotically it was found to make no difference in any of these cases as to which side of the dead membrane was presented toward the solution and which to the pure water. The substances passed through readily in either case. Moreover, all of the substances also passed through vegetable membranes such as apple skins, and the rinds of oranges, lemons, and grapefruit. In all of these experiments the procedure was quite simple, the septum being stretched over a suitable wooden frame so as to form a sort of cup to receive the solution, the outer side of the membrane being bathed with distilled water. However, as already stated, all of these membranes were dead and in the investigation of the treatment of infections by means of boric acid applications the membranes were, of course, alive.

After finishing the above mentioned work with Dr. Ochsner, I concluded to make some further investigations of the passage of dissolved substances, and particularly of boric acid, through perfectly normal living skin. It seemed to me highly desirable to carry on such experiments in order to ascertain whether substances other than boric acid actually pass through the living skin in estimable quantities or not, especially since it is commonly held that the latter is practically impermeable to non-corrosive substances brought into contact with it.

EXPERIMENTAL.

The experiments were performed in a simple manner. Both feet were washed with hot water and a little castile soap, thoroughly rinsed repeatedly with warm, distilled water and finally with 20 per cent alcohol. After being wiped with a clean cloth, they were then immersed to a depth just above the ankle in the solution to be tested. The latter was kept at 45°C. in a 5 gallon stoneware jar which served as the foot bath. The feet were kept resting but lightly, and not flatly, on the bottom of the jar, and they were also moved about occasionally. Samples of urine were taken from time to time, and analyzed. The spectro-scope was employed in testing for lithium, cesium, rubidium, and

strontium. The entire urine was carefully evaporated to dryness and the residue acidified with concentrated hydrochloric acid before bringing it into the flame for the spectroscopic test.

The turmeric paper test was employed in testing the urine for boric acid, the entire sample of urine being made slightly alkaline with pure sodium bicarbonate evaporated to dryness and the residue ignited. The residue was then dissolved in a little water and acidified with concentrated hydrochloric acid. The turmeric paper was then dipped into this solution and dried on a large convex cover-glass on the water bath. The urine may also be acidified directly with concentrated hydrochloric acid, the turmeric paper dipped into the resulting solution and dried on the water bath. Only in this case, the red color developed by the paper has a somewhat darker shade, probably due to the coloring matters in the urine. If the turmeric paper is fresh and of good quality, the red color developed by the boric acid is quite bright and uniform, and the test may be used to make quantitative determinations of small amounts of boric acid. This fact was found early in the investigation. By making a 1 per cent standard solution of boric acid in water and then preparing various lower strengths by appropriate dilutions, acidifying each of the latter with a definite amount of concentrated hydrochloric acid, dipping turmeric paper (2 by 4 cm. in size) into each of these dilutions, and drying the papers on the water bath, a color scale was obtained by means of which satisfactory quantitative estimations could be made. If the urine is made alkaline, evaporated, and the residue ignited as above described before making the color test with turmeric paper, the quantitative determination can be made by comparing with the color scale obtained from the aqueous solutions of boric acid as just mentioned. If the turmeric test is made by acidifying the urine with concentrated hydrochloric acid, dipping in the turmeric paper, and drying the latter, the quality of the red color is a little different, as above stated, so that accurate quantitative determinations cannot be made by comparison with the standards prepared from the aqueous boric acid solutions. In this case, however, the difficulty can be readily overcome by preparing the color standards by dissolving boric acid in normal urine and diluting with the latter instead of distilled water. The sample

of normal urine for this purpose may be obtained from the person experimented upon before the actual experiments are begun. An average sample from a number of normal individuals was also found to suffice for the purpose.

The bladder was emptied an hour before beginning the experiment and neither liquids nor food were taken during the time the feet were being soaked. The results are as follows:

Experiment 1.—The solution consisted of 6 liters of water plus 450 gm. of boric acid and 3 liters of 95 per cent alcohol. The feet were immersed at 2.27 p.m.

2.47 p.m.	The urine contained	0.001	per cent boric acid.
3.08	" " " "	0.0015	" " " "
4.06	" " " "	0.002	" " " "
5.05	" " " "	0.005	" " " "
5.05	Discontinued soaking the feet.		
5.30	The urine contained	0.01	per cent boric acid.
7.00	" " " "	0.005	" " " "
10.00	" " " "	0.002	" " " "
8.00 a.m.	" " " "	0.002	" " " "
9.15	" " " "	0.001	" " " "
11.30	" " " "	0.001	" " " "
2.00 p.m.	" " " "	traces of boric acid.	

Experiment 2.—This was performed to see how soon after immersion of the feet in the boric acid solution that substance would appear in the urine. The experimental conditions were exactly as in Experiment 1. It was found that it required no longer than 5 minutes for the boric acid to make its appearance in the urine.

Experiment 3.—The solution consisted of 6 liters of water plus 1 gm. of each of the following: H_3BO_3 , LiCl , RbCl , CsCl , and SrCl_2 . The feet were immersed from 2.37 to 4.45 p.m. The bladder was then emptied and the urine tested as above described. Not a trace of any of the dissolved substances was found in the urine.

Experiment 4.—The foot bath consisted of a 5 per cent lithium chloride solution. The feet were then immersed for 30 minutes. No lithium was found in the urine.

Experiment 5.—The foot bath consisted of a 10 per cent lithium chloride solution. The feet were immersed for 42 minutes. No lithium was found in the urine. The entire experiment was repeated on the following day with the same result.

Experiment 6.—The foot bath consisted of a 10 per cent aqueous lithium chloride solution to which an equal volume of 95 per cent alcohol had been added. The feet were immersed for half an hour. Not even a spectroscopic trace of lithium was found in the urine.

Experiment 7.—The foot bath consisted of an aqueous solution of $\text{Li}_2\text{B}_4\text{O}_7$ which contained one-sixth of a mol per liter. The feet were soaked from

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10.18 a.m. to 12.30 p.m. Seven samples of urine were taken at various intervals during this time, but not one of them contained even a trace of boric acid or lithium.

The experiment was repeated using $\text{Na}_2\text{B}_4\text{O}_7$ instead of $\text{Li}_2\text{B}_4\text{O}_7$. Again, not even a trace of boric acid was found in the urine.

Experiment 8.—This was like Experiment 7 except that lithium oleate was used instead of lithium borate. Not a trace of lithium was found in the urine.

Experiment 9.—This was like Experiment 7 except that lithium linolate was used instead of lithium borate. Not a trace of lithium was found in the urine.

Experiment 10.—This was like Experiment 7 except that lithium caseinate was used instead of lithium borate. Again, not a trace of lithium was found in the urine.

Experiment 11.—The foot bath consisted of 6 liters of a 10 per cent lithium chloride solution to which 200 cc. of 5 N hydrochloric acid had been added. The feet were immersed at 10.58 a.m. and samples of urine were taken after 5, 10, 20, 30, 40, and 60 minutes. Not even a spectroscopic trace of lithium was found to be present in any of these samples.

The urine before beginning the experiment was normal and faintly acid toward litmus. All five of the samples of urine tested after immersing the feet were slightly alkaline toward litmus; the last sample being decidedly more alkaline than the others.

In this solution the feet felt "hard" when rubbed against each other and not slippery as in the solutions in Experiments 7, 8, 9, and 10 which were, of course, alkaline.

Experiment 12.—The feet were immersed in 0.1 N H_2SO_4 solution for 10 minutes. After 5 minutes the urine was distinctly alkaline toward litmus and after 10 minutes the alkalinity was plainly more pronounced.

The normal faintly alkaline reaction of the saliva remained the same throughout the experiment.

Experiment 13.—The feet were immersed in 0.1 N citric acid for half an hour. Samples of urine were taken every 10 minutes during this time. The urine always remained normally faintly acid. This experiment was done from 3.30 to 4.30 p.m. of the same day as Experiment 11 which was carried on from 10.52 to 11.02 a.m. The feet soaked in 0.5 N citric acid solution actually increased the acidity of the urine. The saliva remained alkaline.

When 1 gm. of citric acid was taken internally by mouth, the urine was perceptibly more acid than normal, in 5 minutes. The same was found to be true when 5 cc. of 0.1 N H_2SO_4 , diluted so as to be N/150, were taken internally.

Experiment 14.—The foot bath consisted of a 17.64 per cent aqueous solution of cane-sugar. The feet were immersed from 1.30 to 5.20 p.m. The sugar solution did not show any perceptible change in content and, of course, no sugar was found in the urine.

DISCUSSION.

A review of the experiments performed shows that of all of the substances studied boric acid, and this alone, passes through the living skin in perceptible quantities. When salts of boric acid like borax or lithium borate are used, no boric radical is found in the urine. It requires free boric acid to pass through the living skin. Again, the amounts of boric acid found in the urine of a normal person whose feet are soaked in boric acid solution are much the same (only a few hundredths of a per cent) as those observed in patients treated with the boric acid compresses for blood poisoning. Why boric acid should possess this peculiar property of passing through the living skin which other substances, including salts of boric acid, are quite unable to penetrate, is hard to say. We are here evidently confronted with a highly specific property of boric acid. The solution of the latter probably is adsorbed by the skin (*i.e.* loosely combined with the latter) and from this loose combination the boric acid is swept away by the blood stream. It is remarkable that in 5 minutes after the feet are immersed in the boric acid solution, boric acid is present in the urine.

When persons suffering from blood poisoning are given boric acid by mouth, relatively large quantities of boric acid are found in the urine; but the infection is not quelled by this treatment. On the other hand, when the boric acid solution is put upon the skin, but very small amounts appear in the urine (though they get there rapidly), and the infection is conquered. Borates (like borax), when used topically in cases of blood poisoning, do not effect a cure, and we can now readily understand this, for these salts are quite unable to permeate the living skin.

All of the substances tested pass readily through dead human skin and also through other dead animal membranes. These substances also pass through the living mucous membranes of the mouth and alimentary canal. Now, since experiments on osmosis have demonstrated conclusively that the chemical nature of the membrane is the determining factor as to whether a substance will pass through the membrane or not, one is led to the conclusion that dead skin and living skin are chemically different, for they act quite differently osmotically. Similarly,

the living mucous membranes are chemically different from the living skin since they act differently osmotically. These latter two septa act alike when dead in that they let through all of the substances studied; but when living, the skin lets through boric acid only, while the mucous membranes are penetrated by all of the dissolved substances.

Whether small amounts of sodium or potassium salts may pass through the skin is hard to determine for salts of these metals are normally present in the body. For this reason, salts of other alkalies not normally present in the body were tested. Lithium seemed particularly suitable for this purpose since it is readily detected in minute quantities by means of the spectroscope. However, in none of the experiments (Nos. 3 to 11 inclusive) was even a trace of lithium found in the urine.

The alkalinity which developed in the urine when the feet were immersed in a hydrochloric acid solution (Experiment 11) or a sulfuric acid solution (Experiment 12) is very interesting. Evidently this stimulus causes reaction in the body to combat, *i.e.* neutralize these acids, which action is strong enough to continue to produce alkalinity. The weaker acid, citric acid (Experiment 13), does not have the same effect as hydrochloric or sulfuric acid. Stronger solutions of citric acid actually cause perceptibly greater acidity of the urine. This peculiar action still awaits adequate explanation. It is hoped to study the matter further, experimentally. However, it is clear that sulfuric, hydrochloric, and citric acids when taken by mouth acidify the urine, while when the skin is bathed with them the first two cause an alkaline reaction of the urine while the citric acid has the opposite effect. In the face of this, it does not seem so peculiar that boric acid also should have quite a different effect when taken internally from that produced when it passes into the system through the living skin.

A CRITICAL STUDY OF THE JENDRASSIK REACTION FOR WATER-SOLUBLE B.

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The biological method for testing the presence of a vitamin in food or in an extract therefrom is one that consumes much time and labor. Consequently, any suggestion which would lessen the time and labor necessary for reaching conclusions as to the presence or absence of vitamin B or any other vitamin would be highly welcomed. Such a suggestion has come from Jendrassik (1), who has reported in this Journal a color reaction specific for water-soluble B.

The reaction according to Jendrassik is carried out as follows: To the concentrated aqueous solution of the preparation to be tested for vitamin B, acetic acid is added to make a 2 per cent solution. Ferric ferricyanide, freshly prepared by mixing equal volumes of tenth molar ferric chloride and tenth molar potassium ferricyanide, is now added. In the presence of water-soluble B, a characteristic blue color is developed, the result of the formation of ferric ferrocyanide or Prussian blue. The ferricyanide in the reagent is reduced to ferrocyanide, which further reacts with the ferric chloride. The reagent is added as long as the depth of the color increases. The tube containing the reaction mixture is stoppered to hinder reoxidation, allowed to stand for 10 minutes, diluted with 1 to 5 volumes of distilled water, and again observed for color. A positive test is indicated by the presence of a distinct blue color or, after standing for some time, of a blue precipitate, while lack of change or the formation of a green color is regarded by Jendrassik as a negative finding.

We have examined a number of phenols, using the exact Jendrassik technique, and were surprised to find that the compounds following gave positive results.

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*Monophenols.*¹—Phenol (5), *o*-cresol (5), *m*-cresol (2), *p*-cresol (3), *o*-xylenol (4), *m*-xylenol (4), *p*-xylenol (4), thymol (4), carvacrol (4), α -naphthol (10), β -naphthol (4).

Monophenols with Alcohol Group.—Diathesin (*o*-hydroxybenzyl alcohol) (1).

Monophenols with Aldehyde Group.—Salicylic aldehyde (7).

Monophenols with Carboxyl Group.—Tyrosine (5), β -hydroxynaphthoic acid (5).

Esters of Phenolic Acids.—Methyl salicylate (5), phenyl salicylate (7).

Monophenols with Halogen.—Tribromophenol (3), diiodothymol (5).

Monophenols with Nitro Group.—*o*-Nitrophenol (8), *p*-nitrophenol (4).

Monophenols with Amino Group.—*p*-Aminophenol hydrochloride (9), monomethyl-*p*-aminophenol sulfate (15).

Ethers of Monophenols.—Phenetol (7).

Diphenols.—Pyrocatechol (6), adrenalin (10), resorcinol (6), orcinol (3), hydroquinone (4).

Ethers of Diphenols.—Guaiacol (5), eugenol (3).

Ethers of Diphenols with Aldehyde Group.—Vanillin (4), piperonal (4).

Ethers of Diphenols with Carboxyl Group.—Vanillic acid (4).

Triphenols.—Pyrogallol (5), phloroglucin (5).

Triphenols with Carboxyl Group.—Gallic acid (8), tannic acid (6).

Glucosides Yielding Phenols on Hydrolysis.—Arbutin (3), esculin (5), salicin (5).

Phenolic Alkaloids.—Morphine (3), codeine (5), apomorphine (8), cotarnin (7).

Practically all the phenols examined, with but few exceptions, gave the test. From a review of the literature we find that the reaction has been recommended as early as 1873 by Hager (2), as a test for the phenolic alkaloid, morphine. Salicylic acid and acetyl salicylic acid yielded a purple color due to the fact that they gave the ferric chloride color reaction in preference to the Jendrassik reaction. Anisole, anisic acid, heliotropine, and heroin were negative, probably as a result of the absence in the molecule of a free phenolic hydroxyl, although the other compounds with a tied-up phenolic group such as phenetol, guaiacol, and eugenol gave positive reactions. The mononitrophenols gave the characteristic blue color, but *m*-dinitrophenol, dinitroaminophenol (picramic acid), and trinitrophenol (picric acid) proved negative. The presence in the molecule of many nitro groups, derived from such a strong oxidizer as nitric acid, exerts a retarding influence upon

¹ The number in parenthesis after each compound shows the length of time in minutes required to boil with alkali in order to render the Jendrassik reaction negative.

the reducing capacity of the phenol, which is no longer able to convert the ferricyanide in the reagent to ferrocyanide. Nitroso- β -naphthol proved negative, while β -naphthol gave a positive reaction.

At first we felt that the test would be specific for phenols, but further work showed us that it proved also positive with certain non-phenolic compounds, such as thiourea, uric acid, paraformaldehyde, *p*-dimethyl aminobenzaldehyde, Michler's ketone, sodium taurocholate, hydroxylamine hydrochloride, benzidine, toluidine, *p*-phenylenediamine hydrochloride, α - and β -naphthylamine.

The compounds that respond negatively to the Jendrassik test were the following:

Organic Acids.—Formic, tartaric, citric, lactic, pyromucic acids.

Aldehydes and Ketones.—Formaldehyde, paraldehyde, trichloroacetaldehyde, cinnamic aldehyde, acetone, chloroacetophenone, ethyl acetoacetate.

Carbohydrates.—Xylose, rhamnose, fructose, glucose, galactose, maltose, sucrose, lactose.

To emphasize the specificity of the reaction Jendrassik took advantage of the fact that water-soluble B is destroyed by boiling with alkali. He found that all his active water-soluble B preparations gave positive results with his reagent, but failed to do so after boiling with sodium hydroxide. We had hopes that the compounds we found to yield positive reactions would continue to respond after treatment with alkali. We held the view that the use of alkali would therefore serve to differentiate water-soluble B from all other compounds that give a positive Jendrassik reaction. To test the validity of this view we studied the effect of sodium hydroxide upon the compounds, both phenolic and non-phenolic, which yielded positive results. Each compound was boiled with 5 per cent sodium hydroxide. The alkaline mixture was subsequently neutralized with acetic acid and finally made up to a concentration of 2 per cent acetic acid. We were again surprised to find that the compounds now reacted negatively. The length of boiling necessary to render the test negative varied with the individual compound.¹ It seems that the destruction by alkali is not only characteristic of water-soluble B but of a host of organic compounds, especially those containing a phenolic hydroxyl.

Jendrassik lays claim to the fact that water-soluble B cannot be a phenol, since he found phenol itself unreactive to his reagent.

Our results on the contrary seem to show that it may very likely be a phenolic compound or at least associated with one. Jendrassik borrows support for his argument against the phenolic nature of vitamin B by stating that all his biologically active preparations failed to give a positive Liebermann test or a positive Millon reaction. It is a known fact, however, that neither the Liebermann (3) nor the Millon (4) reaction is given by all phenols. To satisfy ourselves on this particular point we carried out a number of experiments with the Liebermann and Millon reagents, choosing a few typical phenols for the test.

Phenol, tricesol, pyrocatechol, resorcinol, phloroglucin, salicylic acid, and thymol gave the Liebermann reaction, while hydroquinone and the phenolic alkaloid, morphine, did not. The phenols that reacted positively were boiled with a 5 per cent sodium hydroxide solution. After neutralization and subsequent acidification they failed to give the reaction. A phenol gives a positive Liebermann reaction when, on addition of sulfuric acid and potassium nitrite, there forms a greenish blue or blue color, which on dilution with water changes to red, while making this diluted mixture alkaline with sodium, potassium, or ammonium hydroxide causes the red color to go over into a green. This color sequence—greenish blue or blue-red-green—is not adhered to by the phenols tested after alkali treatment.

A similar set of experiments was carried on with the Millon reagent. Mathews (4) states that the Millon reaction is given by all organic compounds containing a monohydroxy benzene nucleus but not by di- or trihydroxy phenols unless one of the hydroxyls is substituted, as in esters or ethers. We found that phenol, tricesol, resorcinol, phloroglucin, and salicylic acid reacted positively, but that pyrocatechol, hydroquinone, thymol, and morphine reacted negatively. As with the Liebermann test, the positive-reacting compounds yielded negative results after treatment with alkali followed by neutralization and acidification. Instead of the typical red color or precipitate, a yellow precipitate formed. To dispel the impression that the sodium acetate produced by the neutralization of the sodium hydroxide with acetic acid interfered with the Millon reaction tried, we tested out the effect of a 5 per cent sodium acetate solution. The phenols dissolved in it still give a positive Millon reaction.

We wish to extend our thanks to John T. Little and to Michael Gleason, who have painstakingly repeated all the experiments in order to check the findings recorded.

CONCLUSIONS.

1. The ferric ferricyanid^e reaction proposed by Jendrassik is not a specific test for water-soluble B. It is given by phenols and may serve as a test for phenols in the absence of a few positively reacting non-phenolic compounds.

2. Alkali reacts with phenols to give derivatives or fragments that no longer respond to the ordinary phenol tests. Phenols resemble vitamin B with respect to the destructive effect of alkali.

3. The finding that active preparations of water-soluble B do not give a positive Millon or Liebermann reaction is no proof of the fact that vitamin B is not a phenol, since neither the Millon nor the Liebermann reaction is given by all phenols. The work here recorded does not preclude the probability of the presence in the vitamin B molecule of one or more phenolic groups.

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THE OPTICAL PROPERTIES OF SOME AMINO ACIDS.

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(Received for publication, August 2, 1924.)

As far as known to the writer, no systematic study of the optical properties of the amino acids has ever been made. Through the cooperation of the Protein Investigation Laboratory of the Bureau of Chemistry, access was had to many of the known crystalline amino acids. These had been prepared by the usual methods and were considered analytically pure. The study of the optical properties of these acids was made by the immersion method commonly used by crystallographers and mineralogists and more recently successfully applied by Wright¹ and Wherry² to the study of synthetic inorganic and organic crystalline substances.

While most of the amino acids, with the exception of cystine and tyrosine, are readily soluble in aqueous liquids, they fortunately proved to be insoluble in the oily liquids commonly used for the determination of the refractive indices and other optical properties by the immersion method. The liquids best suited for this purpose were found to be mixtures of mineral oil (Squibb's) with $n = 1.49$, monochloronaphthalene with $n = 1.64$, monobromonaphthalene with $n = 1.66$, and, in a very few cases, methylene iodide with $n = 1.74$. These oils were mixed in such proportions that each differed in n from the next by 0.005 and their exact n values determined on a refractometer. Observations were made in yellow light, approximating that of the D line.

¹ Wright, F. E., The petrographic microscope in analysis, *J. Am. Chem. Soc.*, 1916, xxxviii, 1647.

² Wherry, E. T., The application of optical methods of identification to alkaloids and other organic compounds, *U. S. Dept. Agric., Bull.* 679, 1918.

In addition to the optical determinations, photomicrographs were made of the amino acids to illustrate their crystal habit as ordinarily met in the usual methods of analysis.³

The author desires to acknowledge the generous assistance of Dr. D. Breese Jones of the Protein Investigation Laboratory for furnishing the acids for study and to Dr. E. T. Wherry for many helpful suggestions in the preparation of the paper.

Alanine ($C_2H_4(NH_2)COOH$).

Crystal Habit.—When examined under the microscope in ordinary light the material was seen to consist of rods and needles (Fig. 1).

Refractive Indices (D).— $n_\alpha = 1.515$, common and occurring on some rods crosswise; $n_\beta = 1.540$, occurring crosswise on other rods; $n_\gamma = 1.575$, occurring lengthwise on rods. All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction strong, first and second order colors usually shown; extinction straight; elongation +.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Partial biaxial figures rarely shown.

Diagnostic Characters.—The values n_α (1.515) and n_γ (1.575) are most significant for diagnostic purposes.

Aspartic Acid ($CO_2H \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$).

Crystal Habit.—In ordinary light material was seen to consist of irregular fragments (Fig. 2).

Refractive Indices (D).— $n_\alpha = 1.515$; $n_\beta = 1.560$, occasionally on fragments showing one optic axis up; $n_\gamma = 1.630$, occurring very frequently. All ± 0.001 .

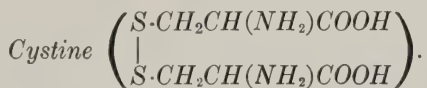
Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction extremely strong, many fragments showing third and fourth order colors; color bands distinctly shown.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—The majority of the fragments extinguish sharply, making it impossible to obtain good interference figures; occasionally fragments show one optic axis up.

Diagnostic Characters.—The most characteristic feature of this substance is the highest refractive index. When immersed in a liquid consisting of a mixture of monochloronaphthalene and mineral oil with $n_D = 1.630$ and examined under the microscope with the polarizer in place, the greater

³ It is not to be assumed that the crystal forms illustrated remain intact in obtaining the optical constants. In many instances it is necessary to crush the material, thereby obtaining irregular fragments.

number of the fragments will match this liquid in one direction. Such fragments show sharp extinction in parallel polarized light with crossed nicols.



Crystal Habit.—In ordinary light the material was found to consist of colorless hexagonal plates and prisms (Fig. 3).

Refractive Indices (D).— $n_\omega = 1.700$, very common; $n_\epsilon = 1.640$, not occurring so frequently as n_ω . Both ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction extremely strong.

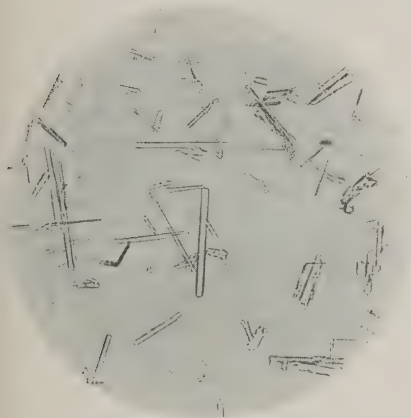


FIG. 1. Alanine. \times about $66\frac{2}{3}$.

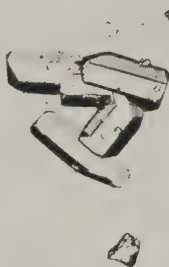
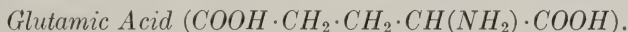


FIG. 2. Aspartic acid. $\times 60$.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Uniaxial figures common; optic sign —.

Diagnostic Characters.—The characters likely to be of greatest value in identifying this substance are the crystal habit and the value of the highest refractive index, $n_\omega = 1.700$. When immersed in a liquid consisting of a mixture of monochloronaphthalene and methylene iodide with $n_D = 1.700$, the hexagonal plates and prisms will completely disappear or show faint orange and blue colors about their margins.



Crystal Habit.—Examination of the powdered material under the microscope in ordinary light showed it to consist of irregular fragments (Fig. 4).

Refractive Indices (D).— $n_\alpha = 1.490$; $n_\beta = 1.605$, occurring on many fragments and diagnostic for the substance; $n_\gamma = 1.620$, occurring on many irregularly shaped fragments. All ± 0.001 .⁴

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction extreme, the colors being second to third order; extinction and elongation indeterminate.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—A partial biaxial figure frequently seen, particularly on fragments showing $n_\beta = 1.605$.

Diagnostic Characters.—All three refractive indices, especially the intermediate index, $n_\beta = 1.605$, are of value in identifying the substance. If immersed in a liquid consisting of a mixture of monochloronaphthalene and mineral oil with $n_D = 1.605$ and examined under the microscope with the polarizer in place, many fragments will be found to match this liquid, at the same time showing faint orange and blue bands of color.

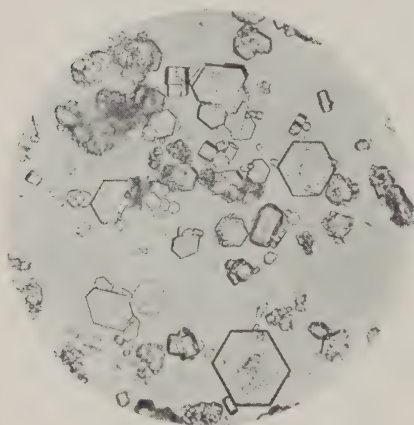


FIG. 3. Cystine. \times about $66\frac{2}{3}$.

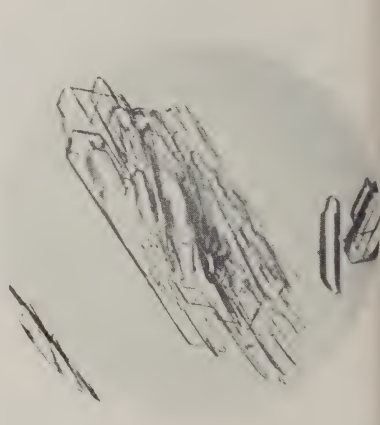


FIG. 4. Glutamic acid. \times about $66\frac{2}{3}$.

Glycocoll (Synthetic) ($\text{CH}_2(\text{NH}_2)\text{COOH}$).

Crystal Habit.—In ordinary light material was found to consist of irregular fragments (Fig. 5).

Refractive Indices (D).— $n_\alpha = 1.495$; $n_\beta = 1.615$, occurring frequently on fragments not extinguished completely and showing a lower index in the other direction; $n_\gamma = 1.650$, not occurring as frequently as n_α and n_β . All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction extreme, brilliant low order colors being quite distinct on most of the fragments.

⁴ Artini, E., *Giorn. Min., crist. e petr. Sansoni*, 1891, ii, 35; also in *Z. Krystall.*, 1894, xxiii, 172.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Biaxial figures common, particularly sections inclined to an optic axis; optic sign —.

Diagnostic Characters.—The most characteristic feature of the substance appears to be the intermediate index $n_\beta = 1.615$ which occurs on fragments remaining bright in parallel polarized light with crossed nicols when the microscope stage is rotated. A liquid consisting of a mixture of mineral oil and monochloronaphthalene with $n_D = 1.615$ will match such fragments.



Crystal Habit.—This material was found to consist of colorless, thin, 6-sided plates, and narrow rod-like plates when examined in ordinary light with the microscope (Fig. 6).



FIG. 5. Glycocoll (synthetic). $\times 34$.

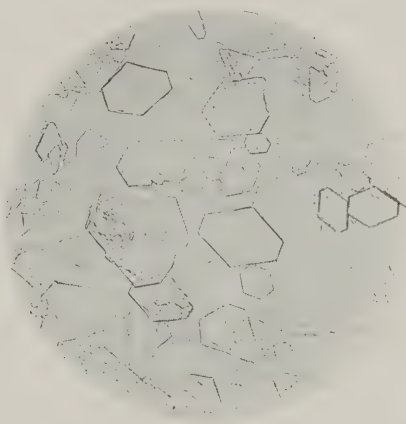


FIG. 6. Leucine. \times about $66\frac{2}{3}$.

Refractive Indices.— $n_\alpha = 1.525$, occurring lengthwise on narrow rod-shaped plates showing negative elongation, not common; $n_\beta = 1.535$, common, occurring usually on plate-like forms showing a higher index in the other direction; $n_\gamma = 1.560$, occurring lengthwise on rods showing positive elongation. All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction strong; extinction straight on narrow rod-like plates; elongation variable.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Partial biaxial interference figures common; axial angle apparently large, indeterminable.

Diagnostic Characters.—The most characteristic feature of this substance is the intermediate value ($n_\beta = 1.535$) for the refractive indices. Immersion liquid consisting of a mixture of mineral oil and monochloronaphthalene and with $n_D = 1.535$ will serve to differentiate the material from the other amino acids.

Phenylalanine ($C_6H_5 \cdot CH_2CH(NH_2)COOH$).

Crystal Habit.—In ordinary light the product was found to consist of 6-sided plates and irregular fragments (Fig. 7).

Refractive Indices (D).— $n_\alpha = 1.600$ which is readily located; $n_\beta =$ approximately 1.610; $n_\gamma = 1.675$, occurring frequently. All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction very strong, polarization colors brilliant, first and second order colors usually shown; extinction apparently inclined.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Partial biaxial interference figures rarely shown.

Diagnostic Characters.—The most diagnostic refractive index for this substance is $n_\alpha = 1.600$ and $n_\gamma = 1.675$, although intermediate value $n_D = 1.610$ may also be used.

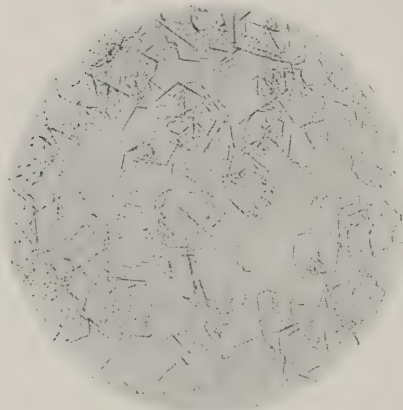


FIG. 7. Phenylalanine. \times about $66\frac{2}{3}$.

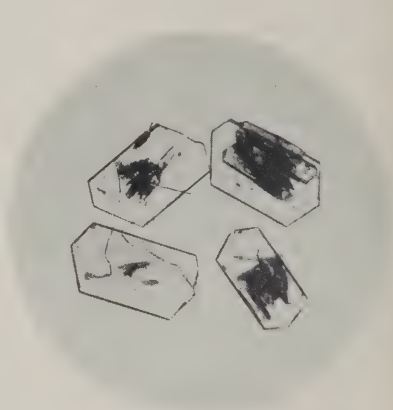


FIG. 8. Serine. $\times 7\frac{1}{2}$.

Serine ($CH_2OH \cdot CH(NH_2) \cdot COOH$).

Crystal Habit.—In ordinary light the powdered material consisted of fragments without any definite form (Fig. 8).

Refractive Indices (D).— $n_\alpha = 1.515$, common and occurring on irregular fragments showing sharp extinction in parallel polarized light with crossed nicols; $n_\beta = 1.575$, occurring very frequently on fragments approximately perpendicular to one optic axis; these fragments remain bright when the stage is rotated with crossed nicols; $n_\gamma = 1.586$. All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction very strong; polarization colors brilliant; extinction and elongation indeterminate.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Partial biaxial interference figures frequently shown, the sign being $-$ and $2E$ apparently large, but not measurable.

Diagnostic Characters.—All three indices are readily located in this substance, the lowest ($n_\alpha = 1.515$) and the intermediate ($n_\beta = 1.576$) being particularly useful for identification purposes. The immersion liquids used consist of a mixture of monochloronaphthalene and mineral oil with the refractive index values as indicated.

Tryptophane ($C_8H_6N \cdot CH_2 \cdot CH(NH_2) \cdot COOH$).

Crystal Habit.—In ordinary light the material was found to consist of very thin plates, rhombs, and irregularly 6-sided crystals (Fig. 9).

Refractive Indices (D).— $n_\alpha = 1.625$, common; $n_\beta = (?)$; $n_\gamma = 1.635$, common. Both ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction weak; crystal fragments colorless.

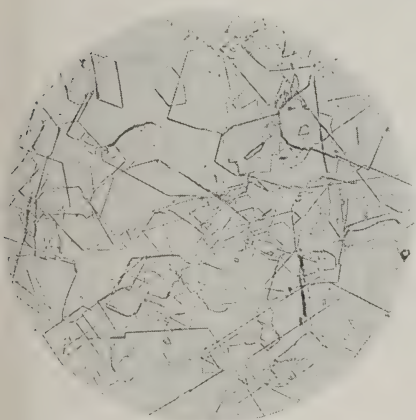


FIG. 9. Tryptophane. \times about $66\frac{2}{3}$.

FIG. 10. Tyrosine. $\times 80$.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—Biaxial figures common; optic sign +.

Diagnostic Characters.—The lowest and highest refractive index values, the thin, papery character of the crystals, and their colorless appearance in parallel polarized light with crossed nicols are the most diagnostic features of this substance. The weak double refraction of the substance quite distinctly characterizes the acid as compared with the other amino acids and, together with the papery nature of the fragments, renders the determination of the index values more difficult.

Tyrosine ($HOC_6H_4 \cdot CH_2CH(NH_2)COOH$).

Crystal Habit.—In ordinary light the material was found to consist of needles and rods (Fig. 10).

Refractive Indices (D).— $n_\alpha = 1.550$, occurring crosswise on needles showing positive elongation; $n_\beta = 1.600$, occurring crosswise; a mean of n_α and n_β , approximately 1.560, is frequent, crosswise; $n_\gamma = 1.680$, occurring invariably on needles lengthwise with positive elongation. All ± 0.001 .

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Double refraction very strong; extinction parallel; elongation +.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—None.

Diagnostic Characters.—The characters likely to be of greatest value in identifying this substance are the crystal habit and the value of the highest refractive index, $n_\gamma = 1.680$. When immersed in a liquid consisting of a mixture of mineral oil and monochloronaphthalene with $n_D = 1.680$, the

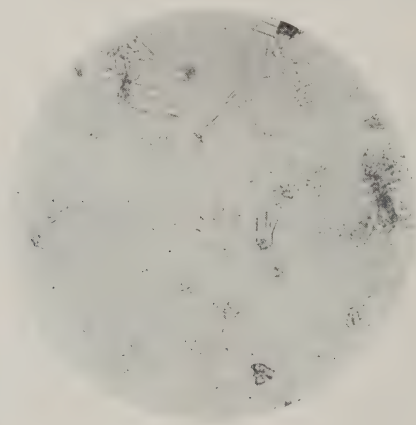


FIG. 11. Valine. \times about $66\frac{2}{3}$.

needles disappear completely when their longest dimension lies parallel to the vibration plane of the polarizer.



Crystal Habit.—In ordinary light the material was found to consist of thin, narrow, rod-shaped plates, characterized by jagged ends and long longitudinal slits (Fig. 11).

Refractive Indices (D).— $n_\alpha = 1.495$, not common; $n_\beta = (?)$; $n_\gamma = 1.565$, very common on jagged rod-shaped plates lengthwise.

Characters Shown in Parallel Polarized Light with Crossed Nicols.—Only low order colors shown; sign of elongation +; extinction, straight.

Characters Shown in Convergent Polarized Light with Crossed Nicols.—None.

Diagnostic Characters.—The crystal habit and the highest index of refraction are the most diagnostic features of this substance. When the material is mounted in a liquid consisting of a mixture of mineral oil and monochloronaphthalene with $n_D = 1.565$, the rod-shaped crystals will be found to match this liquid when they are oriented with their long axis parallel to the plane of vibration of the lower nicol.

TABLE I.

Determinative Table for the Amino Acids.

Immerse crystalline material in liquid indicated and examine under microscope with polarizer in place and diaphragm partially closed.

Immersion liquid.	Remarks.	Amino acid.
(n)		
1.515 (n_α)	$n_\beta = 1.575$ also common.	Serine.
1.535 (n_β)	On plates not extinguishing sharply.	Leucine.
1.565 (n_γ)	Lengthwise on rods.	Valine.
1.575 (n_γ)	“ “ “	Alanine.
1.605 (n_β)	Fragments show orange and blue bands of color.	Glutamic acid.
1.615 (n_β)	Fragments remain practically bright with crossed nicols.	Glycocoll.
1.630 (n_γ)	On many fragments with sharp extinction.	Aspartic acid.
1.635 (n_γ)	— —	Tryptophane.
1.675 (n_γ)	$n_\alpha = 1.600$ also common.	Phenylalanine.
1.680 (n_γ)	Lengthwise on needles.	Tyrosine.
1.700 (n_γ)	Crystals show orange and blue bands.	Cystine.

TABLE II.
Optical Features of Amino Acids Described.

	Alanine.	Aspartic acid.	Cystine.	Glutamic acid.	Glycocoll.	Leucine.	Phenylalanine.	Serine.	Tryptophane.	Tyrosine.	Valine.
Habit.....	Needles.	Plates.	Prisms.	Plates.	Plates.	Plates.	Plates.	Plates.	Plates.	Needles.	Plates.
Indices.											
n_{α}	1.515	1.515	1.640	1.490	1.495	1.525	1.600	1.515	1.625	1.550	1.495
n_{β}	1.540	1.560	1.700	1.605	1.615	1.535	1.610	1.575	(?)	1.600	(?)
n_{γ}	1.575	1.630	1.700	1.620	1.650	1.560	1.675	1.586	1.635	1.680	1.565
$n_{\gamma}-n_{\alpha}$	0.060	0.115	0.060	0.130	0.155	0.035	0.075	0.071	0.010	0.130	0.070
Usual.....	1.575	1.630	1.700	1.605	1.615	1.535	1.675	1.576	1.635	1.680	1.565
Colors.....	1-2	3-4		2-3	1-2	1	1-2	2-3	1	1	1
Extinction.....	Parallel.	(?)	Indeter- min- ate.	(?)	(?)	Parallel.	Inclined. (?)	(?)	(?)	Parallel.	Parallel.
Elongation.....	+	Indeter- min- ate.	"	Indeter- min- ate.	Indeter- min- ate.	Indeter- min- ate.	Indeter- min- ate.	Indeter- min- ate.	Indeter- min- ate.	+	+
Figure.....	0	0	Fre- quent.	Fre- quent.	Usual.	Usual.	Rare.	Fre- quent.	Fre- quent.	0	0
2 E.....	Indeter- min- ate.	Indeter- min- ate.		Indeter- min- ate.	Indeter- min- ate.	Large.	Indeter- min- ate.	Large.	Indeter- min- ate.	Indeter- min- ate.	Indeter- min- ate.
Sign.....	"	"	-	"	-	Indeter- min- ate.	"	-	+	"	"

A PROTEIN FROM THE LEAVES OF THE ALFALFA PLANT.*

BY ALBERT CHARLES CHIBNALL† AND LAURENCE S. NOLAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.)

(Received for publication, September 19, 1924.)

A protein has been prepared from the cytoplasm of the leaves of the alfalfa plant (*Medicago sativa*) by a method recently described for the preparation of spinacin from spinach leaves.¹ Its chemical and physical properties are, in general, similar to this protein, particularly the sensitiveness to the presence of salts when in weak acid solution.

EXPERIMENTAL DETAILS.

The alfalfa in the field stood about 2 feet high and flower buds were about to form. The leaflets were too small to be picked individually, so the top 6 to 8 inches of the plants were cut with a scythe. The leaflets contained approximately 64 per cent of the total solids and 78 per cent of the total nitrogen of the plant cuttings. The fresh alfalfa weighed 5.5 kilos, and contained 1,000 gm. of dry solids or 18.25 per cent and 59.2 gm. of nitrogen or 1.08 per cent. Another sample of alfalfa picked a week earlier had shown that about one-quarter of the total nitrogen belonged to water-soluble non-protein substances.

The vacuole and cytoplasmic proteins were prepared in the same way as the corresponding proteins of spinach.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C. The authors wish to express their thanks to Dr. Thomas B. Osborne for his interest in this work and also for much helpful advice and criticism.

† Seessel Research Fellow, Yale University.

¹ Chibnall, A. C., *J. Biol. Chem.*, 1924, lxi, 303.

Vacuole Protein.

The protein was coagulated by heating the juice, expressed after plasmolysis, to 85° with steam. After washing with alcohol and ether the protein weighed, moisture-free, 2.49 gm. It contained 3.53 per cent of ash, and ash-free 13.22 per cent of N. This protein therefore represents only 0.25 per cent of the leaf solids and 0.56 per cent of the leaf nitrogen. Table I shows the distribution of nitrogen; as in the case of spinach leaves the "amide N" is higher and the "basic N" lower than in the corresponding cytoplasmic protein.

TABLE I.

Showing the Distribution of Nitrogen in the Cytoplasmic and Vacuole Proteins from the Leaves of Alfalfa after Hydrolysis with 20 Per Cent HCl for 16 Hours.

	Cytoplasmic protein.		Vacuole protein.	
	Nitrogen.	Protein.	Nitrogen.	Protein.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide N.....	5.62	0.90	9.97	1.32
Humin N.....	2.85	0.45	2.11	0.28
Basic N.....	25.20	3.97	21.75	2.86
Other N (difference).....	66.33	10.43	66.17	8.76
Total nitrogen.....	100.00	15.75	100.00	13.22

Cytoplasmic Protein.

The protein was flocculated by the requisite amount of acid and allowed to settle overnight. The precipitate, suspended in a volume of 3,800 cc., was redissolved by the addition of 60 cc. of 2.5 N NaOH to give a yellow viscid liquid that could not be filtered even when diluted to 9,000 cc.

The protein was again flocculated by the requisite amount of acid, washed with graded strengths of alcohol, and finally with ether. It weighed 32.3 gm. The alcoholic washings did not appear to contain any appreciable amount of chlorophyll, but the yellow-green color observed in the earlier washings was greatly intensified by the addition of alkali, indicating the presence of pigments such as have already been observed in the

vacuole juice of alfalfa leaves.² The weight of the substances removed by alcohol was 0.2 gm.; only 0.66 per cent of the moisture-free protein. The ether washings were colorless and contained a negligible amount of solids.

Analysis of the Cytoplasmic Protein.

The moisture-free protein contains 15.73 per cent of nitrogen and 0.2 per cent of ash; or, ash-free, 15.76 per cent of nitrogen. The yield of 32.3 gm. therefore represents 3.23 per cent of the leaf solids and 8.61 per cent of the leaf nitrogen. The Molisch test is negative, showing the absence of any carbohydrate impurity. Table II gives the distribution of nitrogen by Van Slyke's method.

Properties of the Cytoplasmic Protein.

When flocculated at its isoelectric point the hydrated protein is almost completely insoluble in water and salt solutions, is coagulated by boiling, and is denatured by strong alcohol. The denatured protein is difficultly soluble in weak alkali. The protein is freely soluble in a slight excess of alkali to give an intense yellow solution which, in a thick layer, appears opaque by transmitted light. In weak acids it is less easily soluble, to give a nearly colorless, but opaque solution. The color of the alkaline solution is probably due to the presence of adsorbed pigments. As noted above alcohol removes these, at any rate, in part. Under conditions that have not yet been fully investigated the protein shows a tendency to gel in both weak acid and alkaline solution.

In weak acid solution the protein is extremely sensitive to the presence of salts; 4 drops of saturated ammonium sulfate solution added to 50 cc. of approximately 0.4 per cent solution of the protein caused complete precipitation at once, while 3 cc. of a 20 per cent solution of NaCl caused precipitation within 3 hours. In weak alkaline solution the protein is also sensitive to the presence of salts; 0.5 cc. of saturated ammonium sulfate or of 20 per cent disodium phosphate added to 50 cc. of approximately

² Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1922, liii, 411.

0.4 per cent protein solution causes slow, but not complete, precipitation on standing. A 0.4 per cent solution of the protein in weak alkali is not precipitated by the addition of an equal volume of alcohol.

Rough colorimetric estimations show that the isoelectric point of the protein lies between pH 4.0 and 4.6. The pH of

TABLE II.

*Van Slyke Analysis of the Cytoplasmic Protein after Hydrolysis with 20 Per Cent HCl for 27 Hours.**

Total nitrogen in aliquots used for analysis was 0.4915 gm.

	Nitrogen.†	Nitrogen.
	gm.	per cent
Amide N.....	0.0271	5.51
Humin N in acid.....	0.0060	1.22
“ “ “ lime.....	0.0072	1.46
“ “ “ amyl alcohol.....	0.0002	0.04
Cystine.....	0.0041	0.84
Arginine.....	0.0753	15.32
Histidine.....	0.0152	3.09
Lysine.....	0.0490	9.97
Amino N in filtrate.....	0.2878	58.56
Non-amino N in filtrate.....	0.0157	3.19
Total N recovered.....	0.4876	99.20

Protein.		
	per cent	per cent
Arginine.....	7.49 (containing N	—2.41)
Histidine.....	1.79 (“ “	—0.48)
Lysine.....	8.17 (“ “	—1.57)
Total.....		4.46

* Nitrogen figures corrected for solubility of bases.

† Mean of two determinations.

the expressed vacuole fluid lies within the same range so that the protein, as in the case of spinacin, was present in the plasmolyzed cell as an anion at a pH very close to that of its isoelectric point.

The usual sensitiveness of this protein to the presence of salts when in weak acid is of great interest in view of the earlier work

of Osborne, Wakeman, and Leavenworth³ on the proteins of the alfalfa plant.

A comparison of their method of preparation with that outlined above leaves but little doubt that their "colloidal precipitate" protein corresponds to the cytoplasmic protein. When first precipitated by alcohol this "colloidal precipitate" contained considerable quantities of calcium phosphate and organic material, which were not completely extracted by their treatment with alcoholic HCl; as shown by the lower percentage of nitrogen (Preparation 6, ash-free, has N = 15.45 per cent⁴), and a faint positive Molisch reaction (unpublished test). It is probable that the insolubility of their protein hydrochloride in water or weak acid, which appeared to them to be an unusual characteristic, was due either to the presence of salts or to the denaturing of the protein by the alcohol used to throw down the original "colloidal precipitate." By treating their crude preparation with hot 60 per cent alcohol containing 0.3 per cent NaOH they obtained a product (Preparation 12⁵) which contained, ash-free, 16.36 per cent of nitrogen, but, as they themselves point out, the protein molecule itself has probably suffered under this somewhat drastic treatment.

The yield of cytoplasmic protein mentioned above, 3.23 per cent of the leaf solids and 8.61 per cent of the leaf nitrogen, does not represent the total amount present in the leaf, as the following two observations will show.

1. Some fresh alfalfa, picked May 26, 1924, was treated by the method of Osborne, Wakeman, and Leavenworth,³ and the clear filtrate heated to 85° with steam. The coagulum of impure protein (N = 13.64 per cent ash-free) contained 15.06 per cent of the total leaf nitrogen. This preparation would include the vacuole protein mentioned above, so that the yield of cytoplasmic protein nitrogen is equivalent to about 14 per cent of the total leaf nitrogen, a proportion similar to that found for spinacin.

2. A batch of 9 kilos of alfalfa, picked July 1, 1924, was treated by the author's method mentioned above. The leaves were

³ Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1921, xlix, 63.

⁴ Osborne, Wakeman, and Leavenworth,³ p. 76.

⁵ Osborne, Wakeman, and Leavenworth,³ p. 81.

plasmolyzed and washed the same day, but the residues remained in the ice chest overnight and were not ground up for the extraction of the protein until the next morning. Very little of the green cytoplasmic material passed into colloidal solution, and after filtering the press-extract the filtrate gave only a small precipitate when treated with acid.

It would appear that some postmortem change takes place in the cytoplasm, possibly a "setting" into a gel of increasing rigidity, which renders it more and more difficult to disperse the cytoplasm into colloidal solution by grinding with water; for the yield of protein is reduced by one-third in 2 hours (the time taken to plasmolyze and wash the leaves used in the present research) and to a negligible amount in 20 hours.⁶ When using this method for the preparation of leaf proteins, then, it is more advantageous to treat the leaves in several small batches rather than as a whole.

⁶ This observation may help to explain a recent criticism of Tottingham, Schulz, and Lepkovsky (Tottingham, W. E., Schulz, E. R., and Lepkovsky, S., *J. Am. Chem. Soc.*, 1924, xlii, 203).

A PROTEIN FROM THE LEAVES OF ZEA MAYS.*

BY ALBERT CHARLES CHIBNALL† AND LAURENCE S. NOLAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.)

(Received for publication, September 19, 1924.)

A protein has been prepared from the cytoplasm of the leaves of ensilage corn (*Zea mays*) by the method recently described for the preparation of spinacin from spinach leaves.¹ Although this protein from corn leaves was not obtained free from carbohydrate, it so closely resembles spinacin and the similar protein from alfalfa leaves as to suggest that it belongs to the same class, and that the carbohydrate was present as an impurity.

EXPERIMENTAL DETAILS.

The leaves, between 3 to 4 feet long, were obtained from a local farm on August 4, 1924, about 1 week before tassels were observed. The thick midrib was torn out, and the leaf material which weighed 6.7 kilos was treated, in approximately two equal portions, as described below.

In a similar batch of leaves picked a week earlier the dry weight of the leaf material used was 19.4 per cent of the fresh weight and the total nitrogen 0.5 per cent. Similarly 26.5 per cent of the solids and 15.5 per cent of the total nitrogen were present in the form of water-soluble non-protein substances.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The authors wish to express their thanks to Dr. Thomas B. Osborne for his interest in this work and also for much helpful advice and criticism.

† Seessel Research Fellow, Yale University.

¹ Chibnall, A. C., *J. Biol. Chem.*, 1924, lxi, 303.

Treatment of the Leaf Material.

The ether method, as used for the preparation of the spinach¹ and alfalfa² proteins, was employed. Experience with the corn leaf, which contains a large proportion of fiber, showed that iced water was necessary to prevent overheating in the meat chopper, with subsequent reduction in the yield of protein. Further, the ether used for the preliminary plasmolysis must not contain any large quantity of alcohol, or the yield of protein is very considerably reduced. However, commercial ether, containing not over 4 per cent of alcohol, is satisfactory.

Vacuole Protein.

The vacuole juice was heated to 90° with steam. No coagulum settled on cooling, so it is assumed that the juice contained no appreciable amount of protein.

Cytoplasmic Protein.

The green colloidal extract of the minced leaves filtered readily through filter pulp, giving a clear reddish brown solution from which the protein was flocculated by adding the requisite amount of hydrochloric acid. In a previous preparation the precipitate had been allowed to settle, the supernatant liquid drawn off, and the protein redissolved in alkali. Thus concentrated, the protein solution filtered with difficulty through paper pulp; no purification of the protein resulted from this second treatment which was therefore not applied in the present case. The protein was washed with graded strengths of alcohol and finally with ether. It weighed 29.6 gm.

Analysis of the Cytoplasmic Protein.

The moisture-free protein contains 13.92 per cent of nitrogen and 3.28 per cent of ash; or, ash-free, 14.41 per cent of nitrogen. The yield of 29.6 gm. of protein therefore represents 2.3 per cent of the leaf solids and 12 per cent of the leaf nitrogen. The Molisch test is positive, showing the presence of carbohydrate. Table I gives the distribution of nitrogen by Van Slyke's method.

² Chibnall, A. C., and Nolan, L. S., 1924-25, lxii, 173.

The humin nitrogen is higher than that found in similar preparations from spinach and alfalfa, probably due to the presence of carbohydrate; the distribution of the basic nitrogen indicates a close relationship between these proteins.

TABLE I.

*Van Slyke Analysis of the Cytoplasmic Protein after Hydrolysis with 20 Per Cent HCl for 28 Hours.**

Total nitrogen in aliquots used for analysis was 0.5338 gm.

	Nitrogen.†	Nitrogen.
	gm.	per cent
Amide N.....	0.0397	7.44
Humin N in acid.....	0.0102	1.91
“ “ “ lime.....	0.0132	2.47
“ “ “ amyl alcohol.....	0.0010	0.19
Cystine.....	0.0041	0.77
Arginine.....	0.0784	14.69
Histidine.....	0.0254	4.70
Lysine.....	0.0468	8.78
Amino N.....	0.2978	55.81
Non-amino N.....	0.0109	2.04
Total recovered.....	0.5275	98.80

* Nitrogen figures corrected for solubility of bases.

† Mean of two determinations.

Properties of the Cytoplasmic Protein.

The properties of this protein are similar to those of the corresponding preparations from spinach and alfalfa. When flocculated at its isoelectric point the protein is almost completely insoluble in water and salt solutions, is coagulated by boiling, and denatured by strong alcohol. It is freely soluble in a small excess of alkali giving a clear reddish brown solution and in a small excess of acid giving a less intensely colored solution. Both solutions are opaque in thick layers, and are sensitive to the presence of salts.

Rough colorimetric estimations show that the isoelectric point of the protein lies between pH 4.0 and 4.6. The reaction of the expressed vacuole fluid lies within the same range so that the protein was present in the plasmolyzed cell as an anion at a hydrogen ion concentration very close to its isoelectric point.

THE TRYPTOPHANE AND CYSTINE CONTENT OF VARIOUS PROTEINS.

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(Received for publication, August 2, 1924.)

Until recently there have been no methods whereby the tryptophane and cystine content of proteins could be estimated so as to give results that would have much more than a qualitative significance. Quantitative values for these two amino acids are therefore lacking in most tables giving the amino acid composition of proteins. During the last few years figures have been published expressing the cystine content of a number of proteins as estimated by the Van Slyke method. It has been shown that cystine is gradually decomposed or altered during the hydrolysis with strong acids. Consequently the figures obtained for this amino acid by the Van Slyke analysis have not been relied upon as representing true quantitative values.

It is well known that the amino acids, tryptophane and cystine, are indispensable for the normal nutrition of animals. It is therefore of interest and importance to have as much information as possible regarding the quantities of these two amino acids in the various proteins of foods and foodstuffs.

Among the recently described methods for the colorimetric estimation of tryptophane and cystine is that of Folin and Looney (1), for the determination of both of these amino acids, and that of May and Rose (2), for the determination of tryptophane. During the course of our investigations on proteins in the Bureau of Chemistry, a large number of proteins have been isolated from various sources, samples of most of which were on hand. This collection afforded an unusual opportunity for applying these colorimetric methods for estimating the tryptophane and cystine content of these proteins, most of which are from common food-

stuffs. In order to make the list more comprehensive, several other proteins from important foodstuffs were specially prepared, and some have been kindly furnished us, acknowledgment for which is made later in this paper.

The cystine determinations were made by the method of Folin and Looney (1) and those of the tryptophane by the May and Rose method (2), with slight modifications. Points of criticism can generally be raised against nearly all methods of analysis in which the results depend upon the comparison of the intensity or shade of color produced by one solution with that of another solution used as a standard. For the estimation of tryptophane and cystine, however, colorimetric methods are by far the best yet published, with possibly one exception (3).

Although we do not claim that all of the figures for tryptophane and cystine given in this paper are absolute, we do believe that they are not far from expressing true values. Furthermore, the fact that the determinations were all carried out by one person under the same conditions gives the results a relative value which is quite independent of their absolute values.

Tryptophane Content of Casein.

The shade of color developed by the reaction of *p*-dimethylaminobenzaldehyde with tryptophane in the presence of the other products of hydrolysis of proteins differs somewhat from that obtained by the reaction of the aldehyde with a solution of pure tryptophane alone, which makes comparative readings unsatisfactory. This difficulty can be overcome by using, instead of a solution of pure tryptophane, a solution of a protein of known tryptophane content as a standard. May and Rose (2) used for their standard of comparison a solution of casein, and made their calculations on the basis that casein contains 1.5 per cent of tryptophane. Few proteins have been subjected to as many tryptophane determinations by different investigators and by different methods as has casein. The results obtained range from 0.5 to over 2 per cent.

Having decided to use casein as a standard in our determinations, our first efforts were directed to ascertain the tryptophane content of casein as determined by the May and Rose method, with the modifications noted in a subsequent paragraph. Several

preparations of casein, prepared in this laboratory from fresh skimmed milk and purified by methods differing somewhat for the different samples, were used. The tryptophane in casein was determined by comparing the color developed with that similarly obtained with zein to which a known quantity of tryptophane had been added before digestion. The zein had been previously found to be entirely free from tryptophane. Several determinations were made, with closely agreeing results, which gave an average value of 2.2 per cent. This figure agrees closely with results which several investigators have recently published, and which were obtained by using different methods. Fürth and Nobel (4), by means of Voisenet's formaldehyde reaction, and using casein from different sources, found from 1.94 to 2.25 per cent, and Fürth and Lieben (5) have reported 2.0 per cent.¹ Thomas (7), using *p*-dimethylaminobenzaldehyde, found 1.7 to 1.8 per cent. Recently Holm and Greenbank (8) have shown that when this aldehyde is used with 20 per cent hydrochloric acid a longer time is required for completion of the reaction than has been generally supposed. By carrying out the determination following Herzfeld's (9) method with slight modifications, and giving sufficient time for the maximum development of the color, they found 2.24 per cent of tryptophane. Practically identical figures (2.2 to 2.3 per cent) are reported in a recent paper by Komm and Böhringer (10). They used a method involving a new modification of the formaldehyde reaction. After the determinations which are presented in this paper were completed there came into our hands the comprehensive article by Onslow (3) in which is described "A method of estimating the tryptophane content of caseinogen, based on determinations of the nitrogen values of the mercuric sulphate precipitate." The tryptophane nitrogen is calculated from the formula:

$$\text{Tryptophane N} = 2 (\text{Monoamino N} - (2/3 \text{ histidine N}) + \text{peptide non-amino N})$$

By this method they found a tryptophane value of 1.93 per cent for English casein, and 2.15 per cent for Merck's casein.

¹ There has come into our hands,—after this paper had been prepared,—a recent publication by Fürth and Dische (6) in which they report a tryptophane content of 1.7 per cent for casein instead of the higher figures formerly found by Fürth and associates.

In Table I are given percentages of tryptophane in casein as reported by various workers. Percentages under 1.5 per cent are not included. It will be seen that from 1.5 to 1.7 per cent of tryptophane has been actually isolated in the form of the crystalline amino acid. Commenting on this fact Onslow states:

"It will be seen that the discoverers of tryptophane actually isolated as much of the crystalline amino acid as most workers claim to have estimated by their methods. Dakin, moreover, by extraction with butyl alcohol, obtained 1.7 per cent at the first attempt, and Onslow (1921) has shown that yields approaching this value may be obtained in routine work.

TABLE I.
Tryptophane in Casein as Found by Various Authors.

Author.	Method.	Tryptophane.
		<i>per cent</i>
Hopkins and Cole (11).....	Isolation.	1.5
Thomas (7).....	Colorimetric.	1.7 to 1.8
Dakin (12).....	Isolation.	1.7
Fürth and Nobel (4).....	Colorimetric.	2.0
Onslow (13).....	Isolation.	1.5 to 1.7
Folin and Looney (1).....	Colorimetric.	1.54
May and Rose (2).....	"	1.5
Holm and Greenbank (8).....	"	2.24
Komm and Böhringer (10).....	"	2.20
Onslow (3).....	Based on nitrogen determinations.	1.94 to 2.15

"The colorimetric methods of Thomas, of Fürth and Nobel and of Holm and Greenbank [and also of Komm and Böhringer]² are the only ones that give values of 1.7% or greater. As there must be considerable loss of tryptophane in the mother liquors after crystallization, it can hardly be doubted that the true value is higher than 2%, and therefore nearer the figures given in this paper and those of Holm and Greenbank."

Since it seems that there can be but little doubt that casein contains at least 2 per cent of tryptophane, we have accepted the value found by us, namely 2.2 per cent, as the percentage of tryptophane in the casein which we used as a standard, and have used that figure as the basis of calculating the values for the proteins given in this paper.

² The words in brackets are ours. The paper of Komm and Böhringer was probably published after Onslow's paper had been written.

Source and Preparation of the Proteins Examined.

Most of the proteins examined were samples of preparations remaining from previous studies, the preparation and properties of most of which have been described in former publications from this laboratory. These include proteins from the following: Chinese velvet bean (14), Georgia velvet bean (15), mung bean (16), navy bean (17), adsuki bean (18), lima bean (19), soy bean (20), Jack bean (21), buckwheat (22), tomato seed (23), squash and cantaloupe seeds (24), kafir (25), wheat bran (26), coconut (27), cohune nut (28), peanut (29), palm kernel, and bark of the locust tree *Robinia pseudacacia*.³ Several proteins were especially prepared for this work, following the methods described by Osborne and associates. These proteins and references to their method of preparation are as follows: Vignin from the cow pea (30); legumin and vicilin from the pea (*Pisum sativum*) (31); legumin and vicilin from the lentil (32); edestin from hemp seed (33); sunflower seed globulin (34); castor bean proteins (33, 35); cottonseed globulin (36); flaxseed globulin (37); zein from maize (38); globulins of the Brazil nut (33); walnut, almond, and filbert (39); proteins of hen's egg (40); and muscle of the ox (41) and halibut (42). The other proteins examined were kindly furnished us, for which we wish to express our thanks: to Dr. R. A. Gortner, for the samples of the prolamins from einkorn, spelt, emmer, sorghum, teosinte, barley, rye, maize, wheat, and kafir, and for a sample of fibrin and casein; to Dr. Thomas B. Osborne for the spinach and alfalfa proteins; and to Dr. George E. Holm for the Witte peptone, blood albumin, and a sample of wheat gliadin.

Methods Used.

The tryptophane determinations were carried out by following in general the method described by May and Rose (2). In their determinations the digestions were made at "35°C. for 24 hours followed by a standing interval for 40 hours at room temperature." Holm and Greenbank have shown (8) that in some cases a longer time is necessary to bring about a completion of the reaction involved. In the work described in this paper it was found that

³ The preparation and properties of the proteins from the bark of this tree will be described in a paper to be published later.

TABLE II.

Percentages of Tryptophane and Cystine Found in Various Proteins.

Protein.	Tryptophane.	Cystine.
	<i>per cent</i>	<i>per cent</i>
Chinese velvet bean (stizolobin).....	1.36	1.55
Georgia velvet bean.		
Total coagulable proteins.....	1.95	2.65
" globulins.....	1.23	1.76
α -globulin.....	2.26	3.26
β -globulin.....	None.	0.66
Albumin.....	2.46	2.69
Mung bean.		
Total coagulable proteins.....	1.62	0.59
α -globulin.....	2.03	1.68
β -globulin.....	1.18	0.44
Albumin.....	2.26	1.34
Navy bean.		
Total globulins.....	1.52	0.59
Phaseolin (or β -globulin).....	0.94	0.58
Conphaseolin (α -globulin).....	2.79	1.53
Adzuki bean.		
Total globulins.....	1.20	0.57
" proteins extracted by alkali.....	1.33	0.71
" coagulable proteins.....	1.32	0.88
α -globulin.....	1.72	1.77
β -globulin.....	0.96	0.41
Proteose.....	None.	2.86
Lima bean.		
Total globulins.....	1.77	0.78
α -globulin.....	1.92	1.56
β -globulin.....	2.16	0.82
Albumin.....	1.37	1.11
Soy bean (glycinin).....	1.66	1.12
Jack bean.		
Total globulins.....	2.29	0.99
Canavalin.....	0.21	0.40
Concanavalin (α -globulin).....	3.36	1.57
Albumin.....	4.30	1.48
Red kidney bean (total globulins).....	1.34	1.00
Naga Uzura kidney bean (total globulins).....	0.94	0.60
Cow pea viginin.....	1.65	0.52
Pea (<i>Pisum sativum</i>).		
Legumin.....	1.76	0.83
Vicilin.....	0.15	0.57

TABLE II—*Continued.*

Protein.	Trypto- phane.	Cystine.
	<i>per cent</i>	<i>per cent</i>
Lentil (<i>Lens esculenta</i> Moensch).		
Legumin.....	0.93	0.68
Vicilin.....	0.68	0.40
Hemp seed edestin.....	2.48	0.97
Buckwheat globulin.....	2.69	2.47
Sunflower seed globulin.....	2.54	1.56
Castor bean.		
Coagulated proteins.....	2.29	1.43
Cottonseed globulin.....	2.58	1.07
Tomato seed.		
Total globulins.....	1.15	1.17
α -globulin.....	1.15	1.20
β -globulin.....	1.45	1.11
Squash seed globulin.....	3.01	1.38
Cantaloupe seed.		
Globulin.....	2.77	1.30
Glutelin.....	3.17	1.11
Flax seed globulin.....	3.98	1.20
Kafir.		
Kafirin I*.....	1.17	0.55
" II†.....	0.73	0.53
Wheat.		
Gliadin (Durum wheat)†.....	1.09	1.42
" I†.....	0.70	1.68
" II†.....	1.09	1.76
Glutenin.....	1.72	1.56
Rye.		
Gliadin I†.....	0.36	2.64
" II*.....	0.75	2.61
Corn, zein.....	None.	0.85
Barley.		
Hordein I*.....	1.05	1.55
" II†.....	0.44	1.47
Oat gliadin.....	None.	3.48
Sorghum prolamins.....	"	0.86
Einkorn ".....	0.47	1.85
Emmer ".....	0.80	1.98
Spelt ".....	1.08	1.79
Teosinte ".....	None.	1.02

* Sample prepared in the Bureau of Chemistry.

† Sample received from Dr. R. A. Gortner.

‡ Sample received from Dr. G. E. Holm.

TABLE II—*Concluded.*

Protein.	Trypto- phane.	Cystine.
	<i>per cent</i>	<i>per cent</i>
Wheat bran.		
Prolamin.....	1.37	2.29
Globulin.....	2.85	1.52
Albumin.....	4.76	3.29
Peanut.		
Total globulins.....	1.00	1.42
Arachin.....	0.88	1.08
Conarachin.....	2.13	3.00
Albumin.....	2.33	1.47
Coconut globulin.....	1.25	1.54
Cohune nut globulin.....	0.65	2.17
Brazil nut excelsin.....	2.59	1.84
English walnut globulin.....	2.84	2.18
Almond globulin.....	1.37	0.85
Filbert “ “.....	2.86	1.50
Palm kernel protein.....	0.74	1.95
Spinach protein§.....	1.85	2.72
Alfalfa “ §.....	2.86	0.93
Wild locust bark (<i>Robinia pseudacacia</i>).		
Globulin.....	3.17	1.28
Albumin.....	4.18	1.03
Hen's egg.		
Ovalbumin.....	2.25	0.88
Conalbumin.....	5.03	3.37
Ovovitellin.....	2.42	0.83
Cow's milk.		
Lactalbumin¶.....	2.69	{ 4.25 3.91
Casein.....	2.20	0.26
Fibrin.....	4.40	3.72
Ox muscle.....	1.25	1.55
Fish “.....	1.25	1.32
Blood albumin.....	1.44	2.88
Witte peptone.....	5.26	1.93
Gelatin.		
Holland.....	None.	0.31
American.....	“	0.15
German.....	“	0.16

§ Dr. Thomas B. Osborne, who furnished these samples, designated them as crude proteins.

¶ The figures given for cystine in lactalbumin are the results of determinations made on two different preparations.

TABLE III.

Comparison of the Cystine Content of Some Proteins as Determined by the Colorimetric and Van Slyke Methods.

Protein.	Colorimetric method.	Van Slyke method.
Navy bean.	<i>per cent</i>	<i>per cent</i>
Phaseolin.....	0.58	0.84
Conphaseolin.....	1.53	1.18
Adzuki bean.		
α-Globulin.....	1.77	1.63
β-Globulin.....	0.41	0.86
Lima bean.		
α-Globulin.....	1.56	1.60
β-Globulin.....	0.82	0.84
Albumin.....	1.11	1.07
Soy bean (glycinin).....	1.12	1.18
Chinese velvet bean (stizolobin).....	1.55	1.20
Georgia velvet bean.		
α-Globulin.....	3.26	1.03
β-Globulin.....	0.66	0.89
Albumin.....	2.69	1.92
Mung bean.		
α-Globulin.....	1.68	1.49
β-Globulin.....	0.44	0.00
Tomato seed.		
α-Globulin.....	1.20	1.28
β-Globulin.....	1.11	1.14
Cantaloupe seed.		
Globulin.....	1.30	1.23
Glutelin.....	1.11	1.07
Squash seed globulin.....	1.38	1.26
Kafirin.....	0.55	0.78
Wheat gliadin.....	1.42	1.17
Barley hordein.....	1.55	1.18
Cohune nut globulin.....	2.17	1.72
Peanut.		
Arachin.....	1.08	0.85
Conarachin.....	3.00	1.07
Cow's milk.		
Casein.....	0.26	0.26*
Lactalbumin.....	4.25	{ 1.73† 3.10‡
Oat prolamín.....	3.48	5.40§
English walnut globulin.....	2.18	1.38¶
Coconut globulin.....	1.54	1.44

* Calculated from results obtained by Van Slyke (Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 531).

† Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 266.

‡ Calculated from results obtained by Crowther and Raistrick (Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434).

§ Calculated from results obtained by Lüers and Siegert (43).

¶ Calculated from results obtained by Mignon (44).

the time required varied with different proteins. Accordingly, after the digestion had proceeded for 24 hours, readings were taken, and the digestion was continued until the color intensity had become constant.

As a check on the method for the tryptophane determination, known quantities of pure tryptophane (2 mg.) were added to zein and the mixtures subjected to the same treatment as was applied to the proteins which were analyzed. The color developed was compared with that of a standard solution of tryptophane. In two determinations, 2.02 and 1.93 mg. of tryptophane were found.

As already noted, calculations of the tryptophane content of the proteins examined were made on the basis that the casein used as a standard contained 2.20 per cent of tryptophane, instead of 1.5 per cent, the figure used by May and Rose.

The cystine determinations were made according to the very satisfactory method of Folin and Looney (1).

The results of the analyses are given in Table II.

DISCUSSION.

In Table III are given for comparison, the percentages of cystine in some proteins, as determined colorimetrically by the authors, and the percentages found for the same proteins by the Van Slyke method. It is generally regarded that the values found by the Van Slyke method are too low, since it is believed that during the acid hydrolysis a decomposition or alteration of the cystine takes place. Van Slyke (45) found that cystine was decomposed or altered by boiling for 24 hours with 20 per cent hydrochloric acid and that only about 50 per cent of the original cystine taken was precipitated by phosphotungstic acid. Hoffman and Gortner (46), on the other hand, found that "cystine is not appreciably decomposed or destroyed when boiled for a long time with 20 per cent hydrochloric acid but is changed into another compound that is optically inactive and has different physical and chemical properties." This isomeric compound was about 2.5 times as soluble in water as the original cystine and its phosphotungstate was about four times as soluble. It is of interest to note the close agreement existing in a large number of cases between the results obtained by the two methods. The outstanding exceptions are the percentages found for conarachin, lactalbumin, the oat prolamin, and the α -globulin of the Georgia velvet bean.

Marked differences exist between the various alcohol-soluble proteins (prolamins). It will be seen that the prolamins as a class are low in tryptophane and rather high in cystine. Zein from corn, and the prolamins from oats, sorghum, and teosinte are entirely lacking in tryptophane. The high percentage of cystine found in the oat prolamins is of particular interest. Although it is not so high as that found by Lüers and Siegert (Table III) by the Van Slyke method, it still exceeds that of most, if not all, other plant proteins.

Of special interest are the percentages of tryptophane and cystine found in the proteins of wheat bran. The tryptophane content of the bran albumin (4.76 per cent) is the highest of that of any plant protein hitherto reported. The globulin is also particularly high (2.85 per cent) in this amino acid. High percentages of cystine were also found in these proteins, especially in the albumin (3.29 per cent). Jones and Gersdorff (26) found that about half of the total proteins isolated from wheat bran consisted of the albumin and globulin in approximately equal proportions. The high content of cystine and tryptophane in these proteins is significant from the standpoint of their nutritive value, and is in marked contrast to that found in the proteins of the wheat endosperm.

A difference is apparent between the globulin of the almond, and that of the English walnut, filbert, Brazil nut, and coconut. This is in accord with results which have been obtained by feeding experiments. Morgan, Newbecker, and Bridge (47) found that the isolated globulins of the almond did not support normal growth in mice when fed at an 18 per cent level in a ration adequate with respect to the non-protein dietary factors. Excelsin from the Brazil nut, on the other hand, has been shown by Osborne and Mendel (48) to be adequate to provide for the normal growth of rats, and Mignon (44), experimenting with mice, found that nearly normal results were obtained with a diet containing as little as 9 per cent of English walnut globulin. The globulin of the coconut has also been shown by Johns, Finks, and Paul (49) to possess satisfactory nutritive properties.

The high regard in which the proteins of the oil seeds, such as flaxseed, cottonseed, hempseed, and nuts, are held, is further justified by their tryptophane content as shown in Table II.

In the figures given for the different bean proteins it will be seen that the α -globulins, as compared with the β -globulins, are characterized by a higher content of both tryptophane and cystine. The tryptophane content of the α -globulins ranges from 3.36 per cent in concanavalin (Jack bean) to 1.72 per cent in the adsuki bean, while in the β -globulins the range is from none in the Georgia velvet bean to 2.16 per cent in the lima bean. The cystine content ranges in the α -globulins from 3.26 per cent (Georgia velvet bean) to 1.53 per cent (navy bean), and from 0.40 per cent (canavalin (Jack bean)) to 0.82 per cent (lima bean) in the case of the β -globulins. It is of interest to note that the β -globulin of the Georgia velvet bean was found to be entirely lacking in tryptophane. This confirms the results found by Johns and Waterman (15). No other vegetable globulin has been shown to lack this amino acid.

The percentage of cystine (4.25 per cent) found in the first sample of lactalbumin was somewhat unexpectedly high. A second preparation was therefore made by a slightly different method. The amount of cystine found (3.91 per cent) was practically the same as that found in the first preparation. As can be seen in Table III, high values for cystine in lactalbumin have also been obtained by the Van Slyke method.

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RESEARCHES ON PROTEINS.

VIII. THE DESTRUCTIVE DISTILLATION OF THE FIBROIN OF SILK.*

By TREAT B. JOHNSON AND P. G. DASCHAVSKY.†

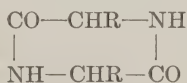
(From the Department of Chemistry, Yale University, New Haven.)

(Received for publication, September 3, 1924.)

Of the numerous attempts to unravel the constitution of the known proteins of silk (fibroin and sericin) the only method that has thus far given useful results is that of hydrolysis. The admirable researches of Emil Fischer and his coworkers in this field of chemistry have revealed the fundamental part that a limited number of amino acids play in the constitution of this natural product. The characteristic linkages which are now considered as expressing the manner of grouping of the amino acids functioning in these proteins are the acyclic polypeptide¹ and cyclic diacipiperazine² constructions as represented in formulas I and II respectively. As sulfur is not present



I



II

in the two proteins found in silk the possibilities of its different types of linkage need not be considered.

* Constructed from part of a dissertation presented by P. G. Daschavsky to the Faculty of the Graduate School of Yale University, June, 1920, in candidacy for the degree of Doctor of Philosophy.

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¹ Abderhalden, E., *Z. physiol. Chem.*, 1923, cxxxi, 281.

² Abderhalden, E., and Stix, W., *Z. physiol. Chem.*, 1924, cxxxii, 238.

It has been reported in a preliminary paper³ that silk noils (crude fibroin) when distilled in a vacuum yield gaseous products, a carbonaceous residue, and a dark red oily distillate of which the last two fractions are formed in about equivalent proportions (40 to 43 per cent). This finding was thought to be worthy of investigation because of the possibility of isolating and identifying characteristic decomposition products of fibroin, and also because of the fact that no previous work of this character has been attempted with silk proteins. In fact, very little is known regarding the nature of the decomposition products which are formed by the destructive distillation of protein material; and what information we have has been contributed chiefly by workers concerned with pyrogenetic decompositions leading to the commercial production of bone oil or Dippel's oil.

The pioneer work on the constituents of bone oil was carried out by Anderson⁴ and later by Weidel⁵ and his coworker, Ciamician.⁶ Summing up their work the principal constituents found were: nitriles of the fatty series, pyrrole and pyridine and their respective derivatives, aliphatic amines, and small quantities of various aromatic and heterocyclic compounds. They conclude that the nitriles are formed by the action of ammonia on fatty acids, that pyrroles are the direct decomposition products of the gelatinous substances of protein nature, and that pyridine and its derivatives probably result by condensation of acrolein, from the breakdown of the fats, with ammonia or primary amines.⁷

The results of research on protein distillation up to 1885 are included in the short contributions by Limpricht,⁸ Williams,⁹ Mills,¹⁰ and a series of papers by Weidel^{7,11} and coworkers in which is reviewed previous work on bone oil and glue.

³ Johnson, T. B., and Daschavsky, P. G., *J. Am. Chem. Soc.*, 1919, xli, 1147.

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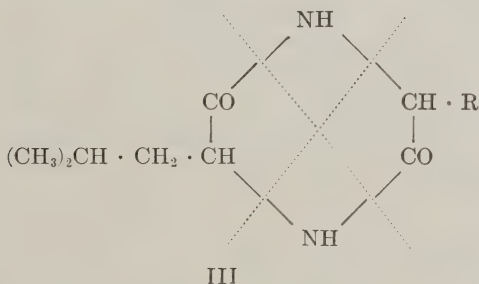
⁸ Limpricht, H., *Ann. Chem.*, 1857, ci, 297.

⁹ Williams, C. G., *Ann. Chem.*, 1859, cix, 127.

¹⁰ Mills, J. *Soc. Chem. Ind.*, 1885, iv, 325; *Chem. Zentr.*, 1886, i, 3.

¹¹ Weidel, H., and Herzig, J., *Monatsh. Chem.*, 1880, i, 1. Weidel, H., and Pick, B., *Monatsh. Chem.*, 1884, v, 656.

Pictet and Cramer¹² sought to obtain primary or intermediate products of decomposition that might furnish some indications of molecular structure of proteins by subjecting ovalbumin to distillation in a vacuum. From the acidic fraction they separated acetic, propionic, normal butyric, and succinic acids, and from the basic fraction an amine to which they assigned the constitution of dihydroaniline. From the neutral portion of the distillate were separated in small quantities acetamide, propionamide, and indole, and in fairly good yield isocaproamide, $(\text{CH}_3)_2\text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CONH}_2$. Weidel and Ciamician⁶ explained the genesis of fatty nitriles in protein distillation by assuming interaction of amines with fatty acids. Pictet and Cramer¹² believe, on the other hand, that these are secondary decomposition products of albumin and result from dehydration of acid amides. In giving an explanation of the formation of isocaproamide the latter investigators actually adopt the diacipiperazine linkage of amino acids in proteins and postulate that the amide need not necessarily be derived from leucine, but that both compounds may result from the same molecular grouping in the albumin complex. These speculations are expressed by formula III, which can break down as indicated giving both leucine and isocaproamide.



As reported in our preliminary paper,³ distillation of fibroin *in vacuo* yields about 43 per cent of its weight in the form of a red oil distillate, 41 per cent in the form of silk carbon or coke, and 16 per cent in the form of gaseous products soluble in alkali and acid solutions. Examination of the red oil distillate revealed

¹² Pictet and Cramer, *Helv. Chim. Acta*, 1919, ii, 188.

the presence of phenol, *p*-cresol, indole, and quinoline. The presence of aliphatic amines in the gaseous fraction was established qualitatively, and the two acids—acetic and propionic—were identified in the form of their corresponding *p*-toluide derivatives. In the basic fraction of the oil distillate positive tests for pyrrole compounds were obtained, but their separation was not possible.

The question at once arises, what is the significance of phenol, *p*-cresol, indole, and quinoline? Phenol and *p*-cresol undoubtedly result from a breakdown of the tyrosine in fibroin and in all likelihood the cresol is the precursor of phenol. The degradation of tyrosine to *p*-cresol and phenol has certainly* been demonstrated in biochemical reactions¹³ as a result of reduction, deamination, and oxidation changes during putrefaction. Whether the mechanism of change from tyrosine to phenol is the same in protein distillation as in protein putrefaction¹⁴ remains to be proven.

To explain the formation of indole is more difficult. Hopkins and Cole¹⁵ have shown that tryptophane when subjected to bacterial action undergoes successive changes to indolepropionic acid, indoleacetic acid, skatole, and finally indole. But, the analytical data produced by Fischer, Abderhalden, and coworkers¹⁶ on fibroin show that tryptophane does not function as a constituent of this protein; therefore, if these data be adopted as a criterion, it follows that this amino acid is not the precursor of indole in silk distillation. Pictet and Cramer¹² also found indole as one of the decomposition products of ovalbumin, but in this case this compound may be assumed to be formed from trypto-

¹³ Baumann, E., *Ber. chem. Ges.*, 1879, xii, 1452; 1880, xiii, 279; *Z. physiol. Chem.*, 1877–78, i, 60; 1879, iii, 250; 1880, iv, 304. Baumann, E., and Brieger, L., *Z. physiol. Chem.*, 1879, iii, 149. Weyl, T., *Z. physiol. Chem.*, 1879, iii, 312. Brieger, L., *Ber. chem. Ges.*, 1877, x, 1027. Salkowski, E., and Salkowski, H., *Ber. chem. Ges.*, 1880, xiii, 191.

¹⁴ Abderhalden, E., *Biochemisches Handlexikon*, Berlin, 1911, iv, 362.

¹⁵ Hopkins, F. G., and Cole, S. W., *J. Physiol.*, 1903, xxix, 451.

¹⁶ Fischer, E., and Skita, A., *Z. physiol. Chem.*, 1901, xxxiii, 177; 1902, xxxv, 221. Inone, R., and Hirasawa, K., *J. Tokyo Chem. Soc.*, 1918, xxxix, 300; *Chem. Abstr.*, 1918, xii, 1702. Abderhalden, E., *Z. physiol. Chem.*, 1922, cxx, 207.

phane, which Hopkins and Cole¹⁷ succeeded in isolating from this protein.¹⁸

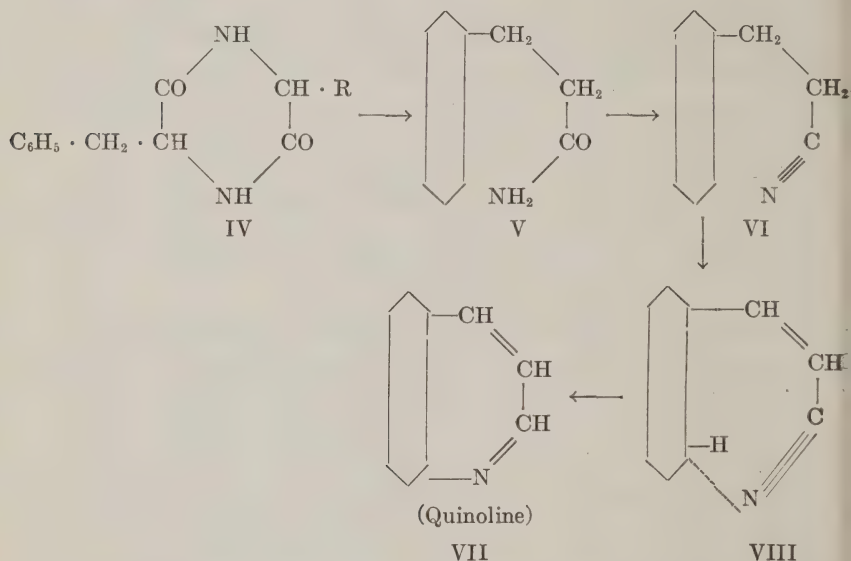
Pictet and Cramer¹² offer no explanation for its formation, merely commenting on the fact that indole is found among the products of protein putrefaction. In fact, with the data at hand it seems somewhat premature to propound any logical theory to explain its formation from fibroin. The conjecture that indole is formed as a result of a secondary reaction of the pyrogenetic-condensation type, becomes plausible when one considers that indole has been made by passing alkylated anilines through a hot tube.¹² Aniline has been shown to be present in bone oil and its occurrence in silk tar is very likely.

To explain the formation of quinoline in fibroin distillation it is possible to make several assumptions, most of which are very probably not tenable. Its genesis must remain unknown until further facts are gathered, but it seems very probable that it results by a pyrogenetic-condensation reaction. As yet we have no conclusive evidence that the quinoline nucleus functions as such in the fibroin molecule, and the following series of reactions are tentatively offered as an explanation of its formation: Phenylalanine, which has been shown to be present in fibroin, may be assumed to occur in the diacipiperazine form IV, and when subjected to the pressure and temperature conditions of destructive distillation in a vacuum undergoes cleavage as indicated to give β -phenyl propionamide V, which, in turn, then loses water to give the corresponding nitrile VI. This nitrile is then converted during the destructive distillation into the nitrile of cinnamic acid VIII, which presents a configuration favorable for ring

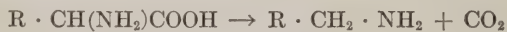
¹⁷ Hopkins, F. G., and Cole, S. W., *Proc. Roy. Soc. London*, 1901, lxviii, 21.

¹⁸ Matsuyama and Mori (Matsuyama, Y., and Mori, T., *J. Chem. Soc. Japan*, 1923, xlv, 377; *Chem. Abstr.*, 1923, xvii, 2718, report the following analytical results for percentages of tryptophane in different proteins: beef protein 1.20, 1.28; swellfish protein 1.38, 1.38; tunny fish protein 1.18, 1.01; salmon protein 1.03, 0.92; scallop protein 1.18, 1.09; serum albumin 1.62, 1.45; fibrin 1.71,—; hemoglobin 1.71,—; egg albumin 1.18, 1.11; fibroin trace, trace; mucin 0,0; gelatin 0,0; globulin 1.62, 1.52; soy bean protein 0.83,—; rice protein 1.33,—; corn protein trace,—; edestin 1.46, 1.40; legumin 0.94, 0.67; conglutin 1.45, 1.28; wheat gluten 1.00, 0.95; zein 0,0. (See also Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 837.)

formation (Baeyer's strain theory). It is then possible to explain the formation of quinoline VII by the attachment of the nitrogen atom of the nitrile grouping to the nuclear carbon atom in *o* position to the side chain; with the subsequent migration of the displaced hydrogen to the unsaturated carbon atom of nitrile VIII. These various changes are expressed by the structural formulas below:



The formation of aliphatic amines is best explained in the usual manner by assuming a normal decarboxylation of the corresponding α -amino acids.



The formation of fatty acids is very probably brought about by the deamination of the corresponding amino acids, a reaction which has many biochemical analogues.



EXPERIMENTAL PART.

Method of Distilling.—As described in our preliminary paper³ the distillation of silk was conducted in a large iron pipe by heat-

ing on an ordinary combustion furnace. With a 1,600 gm. unit of fibroin the operation could be completed in about 8 hours. At the end of the 1st hour's heating under diminished pressure, a pale yellow distillate begins to appear and gradually becomes darker and more viscous as the operation continues. Throughout the distillation gaseous products were produced in abundance, and ammonium carbonate sublimed throughout the condensing tubes and into the wash liquors. The data obtained in seven runs, in which 1,600 gm. of fibroin were used in each unit (total = 11,200 gm.), are recorded in Table I.

TABLE I.
Distillation of Fibroin

Run No.	Red oil distillate.	Silk coke.	Gaseous products absorbed by alkali and acids.*
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	590	640	370
2	650	710	240
3	615	625	360
4	690	700	210
5	830	650	120
6	790	625	195
7	660	650	290
Total.....	4,825	4,600	1,785

* The weights were determined by difference.

The quantity of red oil distillate, therefore, varies from 590 to 830 gm. per 1,600 gm. of fibroin, the yield depending upon the vacuum maintained during distillation and the rate of destructive distillation of the protein.

Identification of Phenol.—A description of our method of separating and identifying this compound has been given in our preliminary paper.³

Treatment of the Red Oil Distillate.—A systematic examination of this distillate, which contained a large proportion of water, was made by dissolving 2,600 gm. of the oil in 2,300 cc. of ether and agitating successively, with 1 liter of 10 per cent sodium hydroxide solution and 2 liters of dilute sulfuric acid, thus finally dividing the distillate into alkali-soluble, acid-soluble, and neutral fractions.

Examination of the Alkali-Soluble Fraction.—The alkali extract obtained above was made neutral with dilute sulfuric acid and the solution concentrated to a small volume by distillation in a partial vacuum. On cooling, we obtained a black tarry product which separated as a supernatant layer. This tar was subjected to distillation, when a profound change seemed to take place, and a clear yellow oil was obtained, boiling from 160–265° at ordinary pressure. On redistillation of this crude product a yellow, mobile liquid was finally obtained, boiling very constantly at 274–280°. This fraction was washed with 10 per cent sodium hydroxide solution, dissolved in ether, dried over pulverized KOH, and finally further purified by distillation when we obtained a pale yellow oil which boiled at 236–239°. This was identified as quinoline, being characterized by its odor and basic character. The picrate of the base was prepared and melted sharply at 203°, and when mixed with some known quinoline picrate no change in melting point was observed. Analysis of quinoline (nitrogen determination):

Calculated for C_9H_7N . N 10.85.

Found. " 10.7.

We did not expect to isolate quinoline from this alkali fraction and its occurrence may be due to one or two causes. Either the extraction of the red oil was not complete or the quinoline was formed as a secondary product during the treatment of the material. The second possibility is the more likely.

Examination of the Acid-Soluble Fraction.—This fraction, when concentrated by heating on the steam bath, became very dark colored. When neutralized with sodium hydroxide a strong odor was noticeable, suggestive of the presence of pyrrole or its derivatives. The pyrrole tests were always positive, but no treatment was found which would enable us to isolate definite substances, the products obtained being bad mixtures which reddened rapidly in acid solution and oxidized and resinified on exposure to the air.

Examination of Neutral Fraction of Red Oil Distillate.—The ether solution of the distillate remaining after thorough washing with alkali and acid was carefully dried over calcium chloride and the ether then distilled off when we obtained a dark red oily

fluid. This was distilled under diminished pressure when the following fractions were collected.

Fraction 1,	boiling at 103–110° at 20 mm.
“ 2,	“ “ 110–120° “ 20 “
“ 3,	“ “ 120–135° “ 20 “
“ 4,	semicrystalline residue.

Fraction 1 (103–110°) was redistilled *in vacuo* and gave a colorless oil boiling at 101–103° at 34 mm. This distillate solidified on cooling and was identified as phenol. In our preliminary work the phenol was separated from the original crude red oil distillate by steam distillation. That phenol was also present in the alkali fraction was evidenced by the odor of the steam distillate and the usual tests characteristic of this compound.

Fractions 2 and 3 (110–120° and 120–135°) were combined and purified by redistillation under diminished pressure when we succeeded in obtaining easily an almost colorless fraction boiling at 125–130° under 21 mm. pressure. This substance was identified as *p*-cresol by the following tests.

It boiled at 200–201° at ordinary pressure, and reacted with ferric chloride giving the bluish coloration characteristic of this compound. Millon's reaction was positive and the *p*-cresol was also easily recognized by its characteristic odor.

Formation of the Phenylurethane, $\text{CH}_3\text{OC}_6\text{H}_4\text{O} \cdot \text{CO} \cdot \text{NHC}_6\text{H}_5$.—5 gm. of the pure distilled *p*-cresol, 2.5 gm. of phenylisocyanate, and a small amount of AlCl_3 as catalyst were heated for 16 hours at 104°. The semisolid reaction product was extracted with ether to separate the suspended AlCl_3 and the solvent then evaporated when the urethane was obtained in a crystalline condition. It was purified for analysis by crystallization from 95 per cent alcohol and melted¹⁹ at 112.5–113.5°. Nitrogen determination (Kjeldahl):

Calculated for $\text{C}_{14}\text{H}_{13}\text{O}_2\text{N}$.	N 6.16.
Found.	“ 6.3, 6.15.

Examination of Fraction IV (Semicrystalline Residue).—This was redistilled under diminished pressure when a thick, viscous fraction was separated, boiling at 195–205° at 24 mm. As puri-

¹⁹ Leuckart, R., *J. prakt. Chem.*, 1890, xli, 319.

fication was not affected by further distillation the oil was finally subjected to steam distillation to remove indole. This was separated from the aqueous distillate by extraction with ether when the indole was obtained in a solid condition after evaporating the solvent. The substance gave an intense, deep red stain when the pine-splinter test was applied, and responded at once to Ehrlich's color test for indole when allowed to interact with *p*-dimethylaminobenzaldehyde in alcoholic hydrochloric acid solution. To a drop of the indole dissolved in 5 cc. of water were added 3 drops of nitric acid and 1 drop of a 6 per cent solution of potassium nitrite when an orange-red coloration developed immediately followed by the deposit of the characteristic red precipitate of nitrosoindole nitrate.²⁰

To an aqueous solution of the indole was added a saturated solution of picric acid and the solution concentrated and cooled. The picrate of indole separated in a crystalline condition in the form of red, scintillating needles melting at 175° to a dark liquid. When mixed with some pure indole picrate the melting point was not altered. The indole was also characterized by its strong fecal odor when its solution was warmed.

Gaseous Products Soluble in 10 Per Cent Sodium Hydroxide.—An aliquot portion of the alkaline solution of the volatile acid products of destructive distillation of fibroin was evaporated nearly to dryness after neutralizing the excess of alkali with dilute H_2SO_4 , and the resulting solution then extracted successively with alcohol and ether. After heating to remove the organic solvents the residue left behind was then fractionally distilled at ordinary pressure when we succeeded easily in obtaining a colorless fraction boiling from 115–150°. This was distinctly acid in character with a sharp penetrating odor, and on fractional distillation was separated further into two fractions boiling at 117–125° and 135–145°, respectively. The lower boiling fraction was chiefly acetic acid which was identified by the preparation of aceto-*p*-toluide as recommended by Mulliken.²¹ This melted sharply at 148° as recorded in the literature.

²⁰ Nencki, M., *Ber. chem. Ges.*, 1875, viii, 722.

²¹ Mulliken, S. P., A method for the identification of pure organic compounds, New York and London, 1911, i, 81.

The fraction boiling at 135–145°, when treated in a similar manner with *p*-toluidine, gave the corresponding *p*-toluide of propionic acid melting²¹ at 123–124°. Higher homologues of these fatty acids were undoubtedly present in the original distillate, but methods were not available for purifying so that we could definitely identify them.

Gaseous Products Soluble in 25 Per Cent Sulfuric Acid.—An aliquot portion of the acid-wash liquor was made alkaline with sodium hydroxide and the solution distilled at ordinary pressure. A strong ammoniacal distillate was obtained which was also characterized by its fishy, amine odor. Several attempts were made to separate the aliphatic amines from ammonia in the form of their salts or characteristic derivatives, but no clean-cut separation could be effected. The pronounced carbylamine and Rimini reaction (violet-red coloration of primary aliphatic amines with acetone and sodium nitroprusside) given by the alkaline distillate, together with the characteristic amine odor and extreme volatility indicated the presence of both methyl and ethyl amines.

SUMMARY.

1. A review is given of the early literature dealing with the destructive distillation of proteins.

2. Fibroin of silk has been subjected to destructive distillation in a vacuum and the following degradation products have been identified: phenol, *p*-cresol, indole, quinoline, ammonia, aliphatic amines (methyl and ethyl (?)), acetic and propionic acids, pyrrole, and carbon dioxide.

3. Fluid and gaseous products of destructive distillation are produced equivalent to about 59 per cent of the fibroin, the remaining 41 per cent being left behind in the still in the form of amorphous carbon or silk coke.

FAT-SOLUBLE VITAMINS.

XIX. THE INDUCTION OF CALCIFYING PROPERTIES IN A RICKETS-PRODUCING RATION BY RADIANT ENERGY.*

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PLATE 1.

(Received for publication, August 8, 1924.)

In a previous paper from this laboratory (1) it was shown that growth-promoting and bone-calcifying properties can be induced in a ration by the simple expedient of exposing it to light. This reaction has now been studied in considerable detail, paying especial attention to the nature of the activation, the kinds of materials that can be activated, the conditions under which these materials can be activated, and the persistence of the activation. Before presenting the data dealing with these studies in detail it appears necessary to submit further evidence in support of the main thesis pointing out the similarity in results obtained by the feeding of cod liver oil, irradiated ration, and by direct irradiation of animals. It is the purpose of the present communication to present histological evidence showing that a rickets-producing ration can be made antirachitic by exposure to ultra-violet light. This supplements our work of the previous paper where we fed a ration, which while never producing the histological or radiographic picture of rickets, yet did not allow normal calcification or growth to take place except with the addition of cod liver oil or the exposure to ultra-violet light of ration or animals.

We believe that in the study of the fundamental causes of rickets too much emphasis has been placed heretofore upon the production

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of the histological picture of rickets characterized primarily by the overproduction of osteoid and the disorderly occurrence and arrangement of various bone elements in a wide metaphysis. This is produced by a peculiar combination of factors as yet imperfectly understood except that growth is a prerequisite for its production. Physiologically, however, there is operative here, among other factors, the same agency which is responsible for the failure of assimilation of calcium in the mature goat (2), in the growing chicken (3), and in the pig (4) as observed in this laboratory; and that agency is radiant energy either acting directly upon certain compounds in the animal body or else acting indirectly through food materials later ingested. In this paper evidence dealing only with the deposition of calcium salts in the rachitic metaphyses will be presented, reserving the discussion of the relation of growth for a later communication.

EXPERIMENTAL.

In order to demonstrate that an irradiated ration will bring about deposition of calcium salts, when a non-irradiated ration will not, it is necessary to have available a ration which will produce a wide rachitic metaphysis in animals without fail. Such rations have already been described in the literature. Sherman and Pappenheimer (5) have reported that they were able to produce pronounced symptoms of rickets in rats by keeping them on a ration of patent wheat flour 95, calcium lactate 2.9, sodium chloride 20, and iron citrate 0.1. McCollum, Simmonds, Shipley, and Park (6) have claimed to obtain the same results with a ration composed of wheat 33, corn 33, gelatin 15, wheat gluten 15, sodium chloride 1.0, and calcium carbonate 3.0. By gross inspection of the probable merit of the two rations for nutritive value,—outside of those conditions with which one is directly concerned in rickets—it appeared to us that the McCollum ration was probably the most satisfactory, especially with the modification that it be specified that the corn used is to be yellow corn. One of the difficulties in producing rickets in rats is the prevention of ophthalmia and infections of the respiratory tract for a long enough time to allow complete exhaustion of the reserves of the antirachitic factor to take place. As vitamin A and the antirachitic vitamin occur closely associated in nature it is not a small matter to provide

plenty of the one and none of the other. With our animals this was an especially important matter because while our young, as standardized, contain a sufficiency of vitamin A in reserve to meet all ordinary requirements they do not contain the large excess desirable for the production of severe rickets. With yellow corn instead of white corn an early impending failure due to an insufficiency of vitamin A could possibly be sufficiently postponed to allow the development of severe rachitic lesions. Upon preliminary experimental trial we found, as expected, that the McCollum ration containing yellow corn was superior to the Sherman ration for the production of rickets with our rats. It accordingly was used in the following experiments.

The rats used were white and white and black pied rats taken from four litters at an age of 24 to 36 days and weighing from 43 to 115 gm. The experiments were run at different times dating from the middle of March. In the first series there were used Litters 1 and 2, in the second series Litter 3, and in the third and in the last series Litter 4. All the animals were kept in our standard rat cages—one litter to a cage except where only two animals or less were kept on the same ration,—then they were kept in a cage formed by dividing our ordinary cage in half by means of a screen partition. Later when the animals were changed to the irradiated ration or otherwise, or when they were kept as controls all animals were segregated to make it possible to obtain consumption records. Distilled water was provided and the cages were bedded with pine shavings.

Sooner or later, and usually very uniformly in a litter, all the rats gave evidence of rickets as indicated by a peculiar flat-footed shambling gait with a decided disinclination to "sidestep" as the rat usually does when suddenly disturbed. Oftentimes they were observed to lie comfortably curled up on their backs, feet in the air, in an apparent attempt to relieve the limbs of the pressure of the body weight. Invariably the animals were plump and exceedingly "well behaved."

The distribution of most of the animals between the two rations is brought out in Table I. In addition, however, six animals taken from Litters 1 and 2 were put upon other control rations or were subjected to irradiation. Two from each litter were put upon the McCollum ration, designated by him Ration

3143, supplemented with 2 per cent of cod liver oil; two were put upon our stock ration (7) supplemented with whole milk and 5 per cent butter fat; and two on Ration 3143 with a 10 minute daily exposure, except Sunday, to ultra-violet light.

The ultra-violet light used was strictly speaking not ultra-violet light; it was the light given off by the quartz mercury vapor lamp which consists of ultra-violet as well as other rays. The lamp used was of the Cooper-Hewitt BY type, run at a burner voltage of 40 and amperage of 4.5. The animals or ration were exposed to this at a distance of approximately 2 feet. The ration when exposed was spread out in a thin layer in 10 gm. quantities in a Petri dish and exposed daily for a period of 30 minutes. After exposure it was transferred to tin cups in which it was offered to the animals. Unconsumed residues were weighed back daily and discarded.

Giving consideration, first of all, to the controls not mentioned in Table I, it was found that Ration 3143 supplemented with 2 per cent of cod liver oil did not give as satisfactory growth as our butter fat-supplemented stock ration. Started at 43 and 57 gm. in weight, a male and a female weighed 62 and 92 gm. in 77 and 91 days, respectively. On the stock ration two rats, a male and a female, from the same litters and started at 47 and 50 gm. weighed 305 and 220 gm., respectively, in the same period of time. Again two other rats entirely comparable to the above in ancestry, sex, and period of time on the ration, started at 55 and 48 gm. weighed 99 and 82 gm. when irradiated.

Histological examination of the distal ends of the radii and ulnæ revealed the absence of a rachitic metaphysis. The histological examinations of these as well as of the bones of the following rats were made after staining with a silver nitrate solution either in gross after splitting the ends of the bones longitudinally or else after sectioning with a microtome in frozen or celloidin block.

In Table I are shown the data obtained with rats which were started on Ration 3143 and then with the development of severe rickets were changed to the irradiated ration and kept on this for from 3 to 19 days. Others were continued on the original ration to serve as controls, at least one for each litter, and in one case the ration was supplemented with cod liver oil to the extent of 2 per cent of the weight of the ration.

TABLE I.

Group No.	Litter No.	Rat No.	Sex.	Age when started. days	Age when changed. days	Final age. days	Weight when started. gm.	Weight when changed. gm.	Final weight. gm.	Ration fed with average daily consumption after change or continuation as control.	Calcium deposition.
1	1	329	♂	24	(74)	81	47	(92)	95	3143 (not changed), 6 gm.	0
1	2	330	♂	24	74	80	53	98	90	3143 + 2 per cent cod liver oil, 4 gm.	++
1	1	331	♀	24	74	80	45	75	65	3143 irradiated, 3 gm.	+
1	2	332	♂	24	74	80	54	110	105	3143 " 6 "	+
1	1	333	♀	24	74	83	47	98	94	3143 " 5 "	++
1	2	334	♂	24	74	95	52	105	99	3143 " 5 "	++
2	3	451	♂	35	63	66	82	127	132	3143 " 8 "	++
2	3	452	♂	35	66	73	87	117	114	3143 " 4 "	++
2	3	453	♀	35	(66)	73	75	(115)	117	3143 (not changed), 7 gm.	0
3	4	494	♂	36	61	68	115	150	148	3143 irradiated, 8 gm.	++
3	4	495	♀	36	61	71	87	105	105	3143 " 6 "	++
3	4	496	♂	36	61	71	110	157	150	3143 " 9 "	++
3	4	497	♀	36	(61)	68	98	(122)	132	3143 (not changed), 7 gm.	0
3	4	498	♂	36	(61)	71	105	(147)	155	3143 (") 9 "	0
3	4	499	♂	36	(61)	71	95	(137)	140	3143 (") 8 "	0

Consumption records were kept daily and averaged for the period following the change of ration, or, in case of the controls, dating from the time that the other rats of the litter were changed. As the table shows the average consumption varied from 3 to 9 gm. per rat per day and it is to be added that in no case did marked decrease of consumption of ration occur during the last few days of the individual trials. It is also to be noted that in no case did any of the control animals consume any more ration than some of those whose ration was changed. Attention is called to this in particular because it is a well recognized fact in pediatrics and has been emphasized by McCollum, Simmonds, Shipley, and Park (8) experimentally, that fasting has a beneficial effect upon infantile rickets. The possibility of fasting having produced our positive results must therefore be eliminated. Shipley, Kinney, and McCollum (9), in attempting to determine the anti-rachitic vitamin content of a number of oils and plant materials, used body weight as an index to whether or not their animals were fasting completely or partially and found that some of their animals showed no signs of healing of the rachitic process in spite of the fact that they had lost considerably in weight. It will be noted in our experience that of all the animals changed to the irradiated ration or supplemented with cod liver oil only one rat, *viz.* Rat 451, did not lose in weight; all our controls, on the other hand, actually gained in weight. We believe that much is to be gained by a detailed study of the relation of growth to rickets and in the meantime it probably is safer to study the relations from the standpoint of food consumption as growth inhibition can be brought about by what safely can be called a physiological reaction to dietary conditions. In these present experiments of ours the situation is rather complicated due to the fact that eight of the rats, and possibly nine, gave the first indications of an impending vitamin A deficiency. This was noted by a slightly unclean condition of the eyes and the merest trace of bareness of the eyelids which foreshadows the incidence of an ophthalmia. Under ordinary observations these slight indications usually escape notice because the animal is still normal in its reactions, it may be and usually is, still gaining in weight and the eyes are not inflamed,—often, in fact, they are quite “beady.” The physiological reactions which we have in mind will be discussed in a later communication.

Whatever may be the significance of the weight relations the histological examination of radii and ulnæ showed that the rats changed to Ration 3143 plus cod liver oil and all the others changed to the irradiated ration, except Rat 451, showed that calcium deposition had taken place. The controls were entirely negative though run for the same period of time and taken from the same litters.

Rat 451 presented an interesting case because of its failure to show calcium deposition and at the same time, like the controls, it gained in weight. It is to be noted that it had received the irradiated ration for a limited time, only 3 days when it was killed, to which, no doubt, can be attributed its aberrant reaction.

Photographic evidence of the deposition of calcium salts resulting from the feeding of the irradiated ration is presented in Plate 1, Figs. 1 to 4. It shows sections of the distal ends of radii and an ulna (Fig. 1) stained with AgNO_3 . Fig. 1 was taken from Rat 451 which, although it had received the irradiated ration for 3 days, showed no deposition of calcium salts in the wide rachitic metaphysis. It, in every way, portrays the picture obtained from control animals. Fig. 2 taken from Rat 494 shows a narrow line of calcium deposits which represents the result of subsistence of the rats on their radiated ration for 7 days. In Rat 495 (Fig. 3) this line had been materially widened which undoubtedly was due to its longer period on the ration. It had been fed on this ration for 10 days. In Rat 334 (Fig. 4) almost complete calcification of the metaphysis had taken place at the end of 21 days. Attention is called specifically to the nature of the calcium deposits in this section. Except for a narrow fringe proximal to the epiphysis the calcium deposits are no longer of the delicate nature characteristic of preliminary calcification; they represent permanent deposits with definitely formed trabeculae.

SUMMARY.

Evidence dependent upon histological methods shows that a ration which induced rickets in the rat can be made definitely antirachitic by the simple expedient of exposing it to ultra-violet light.

The authors wish to express their appreciation of the technical assistance of Miss Alice Outhouse in the preparation of the sections.

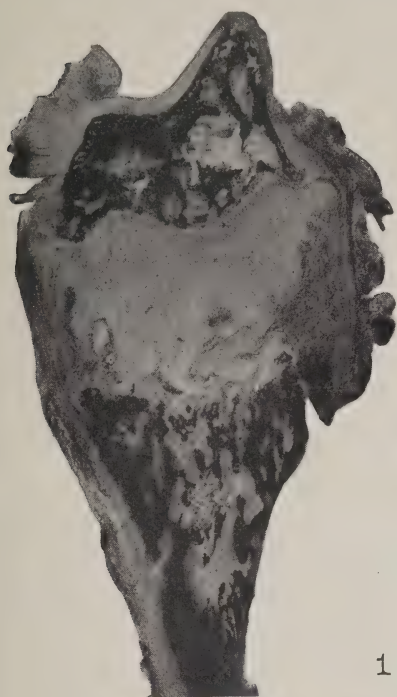
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EXPLANATION OF PLATE 1.

Sections of the distal ends of bones of rats changed from a rachitic ration to rachitic ration irradiated.

- FIG. 1. Rat 451. 3 days on irradiated ration.
FIG. 2. Rat 494. 7 days on irradiated ration.
FIG. 3. Rat 495. 10 days on irradiated ration.
FIG. 4. Rat 334. 21 days on irradiated ration.



1



2



3



4

(Steenbock and Nelson: Fat-soluble vitamins. XIX.)

COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

VII. FATE OF *p*-CHLORO, *p*-BROMO, AND *p*-AMINO ACIDS IN THE DOG, THE RABBIT, AND MAN.

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We have previously shown that several of the derivatives of phenylacetic acid when fed to human subjects do not combine with glutamine as might be expected (1), but either pass through the organism unchanged (2) or else suffer the same fate as when compounds are fed to lower animals (3).

The present work deals with *p*-chlorophenylacetic acid, *p*-bromophenylacetic acid, and *p*-aminophenylacetic acid. These compounds were fed to human beings as well as to dogs and to rabbits, and the differences in metabolic behavior were compared.

Friedmann and Maase (4) fed *p*-chlorophenylalanine to a dog and found that *p*-chlorophenaceturic acid was excreted in the urine. We fed *p*-chlorophenylacetic acid to human beings and to dogs and isolated *p*-chlorophenaceturic acid from the urine, but always found that the product obtained from the human urine was mixed with an additive urea compound. We therefore synthesized the *p*-chlorophenaceturic acid and fed it to a human being, obtaining the same admixture of an additive urea compound.

The rabbit on the other hand excreted the *p*-chlorophenylacetic acid unchanged, both after feeding and injecting.

p-Bromophenylacetic acid after feeding was combined with glycocoll both in the case of man and of the dog, being excreted as *p*-bromophenaceturic acid. The rabbit, however, excreted the substance unchanged. *p*-Bromophenaceturic acid, which had

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not been known previously, was prepared in the laboratory according to the method of Curtius as well as by the Schotten and Baumann reaction. Other new compounds prepared in connection with this substance were *p*-bromophenylacetyl chloride, together with the amide, the cyanide, the ethyl ester, and the silver salt of *p*-bromophenaceturic acid.

p-Aminophenylacetic acid when fed to human beings was found to be acetylated. This acetylated aminophenylacetic acid was also found in the urine of the rabbit after the feeding of *p*-aminophenylacetic acid, but there it is nothing new, for Cohn (5) as well as Ellinger and Hensel (6) have previously demonstrated the ability of the rabbit to acetylate *m*- and *p*-aminobenzoic acids. The dog detoxicates the *p*-aminophenylacetic acid by combining it with glycocoll to form *p*-aminophenaceturic acid, identical with a product synthesized by Hotter (7) many years ago. We then synthesized the *p*-acetylaminophenylacetic acid and fed it to a dog in order to determine its toxicity, and also to see if the animal would detoxicate it by combining it with glycocoll as he had done with the unacetylated *p*-aminophenylacetic acid. The acetylated amino acid was not toxic to the dog, but was excreted within a remarkably short time and uncombined.

Of the three compounds fed, the *p*-aminophenylacetic acid is the least toxic, while the *p*-bromophenylacetic acid is toxic only in relatively large doses. The average human adult may ingest without discomfort a dose of 5 to 8 gm. of the *p*-aminophenylacetic acid, while 3 or 4 gm. of the *p*-chloro compound is sufficient to cause quite a severe headache with perhaps nausea and vomiting. A dose of 2 gm. of the *p*-bromo acid is the maximum for the average adult.

EXPERIMENTAL.

4 gm. of *p*-chlorophenylacetic acid were given to a dog of 18 kilos body weight. The urine was collected by means of a metabolism cage for a period of 48 hours. At the end of this time the dose was repeated, after which the animal was allowed to rest for several days. The urine was evaporated on the water bath to a thick syrup. After cooling, the syrupy mass was acidified to Congo red with dilute sulfuric acid, placed in a rotary

extractor, and thoroughly extracted for 3 hour periods with ethyl acetate. After feeding a total of 12 gm. of *p*-chlorophenylacetic acid, 8.7 gm. of *p*-chlorophenaceturic acid were isolated from the urine. After two recrystallizations from hot water the substance melted quite sharply at 165°C., and gave the following results for nitrogen by Kjeldahl method.

Found. 6.13 and 6.14 per cent.

Theoretical. 6.15 per cent.

The *p*-chlorophenylacetic acid was next ingested by a man in doses of 2 and 3 gm. on every 2nd day, until a total of 11 gm. had been taken. The urine was treated in exactly the same way as described above for the dog. The yield, however, of any detoxication product was always very poor, and the substance isolated invariably melted, after one or two recrystallizations, at 133–140°C. It seemed possible that a glutamine compound might be present, but analysis showed the presence of only 1 nitrogen atom in the molecule. After repeated recrystallizations from water it was possible to raise the melting point to 159°C. The substance was then hydrolyzed by boiling it with concentrated hydrochloric acid under a reflux condenser. The hydrolysate was extracted repeatedly with ether, the ether evaporated to dryness, and the residue taken up in hot water. On cooling, *p*-chlorophenylacetic acid crystallized out and was identified by its melting point. From the acid solution, glyco-coll was obtained and identified by its conversion into the hydrochloride of its ethyl ester.

Two ways were eventually found for purifying the crude product obtained from the urine, both methods being based on the removal of the urea from the additive compound of urea with *p*-chlorophenaceturic acid. The better of these methods is simply to make the cold, water solution alkaline with barium hydroxide. This splits off the additive urea. Carbon dioxide is then passed through the alkaline solution for several hours to precipitate the excess barium. After filtering off the barium carbonate, the solution is slowly evaporated to dryness on the water bath and the dried residue extracted with boiling *absolute* alcohol. (Urea is very soluble in hot absolute alcohol, while the barium salt of the *p*-chloro acid is very insoluble.) In order

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to obtain the pure acid it is merely necessary to remove the barium with dilute sulfuric acid, and concentrate the solution until the *p*-chlorophenaceturic acid crystallizes out. After one or two recrystallizations the compound melts at 164–165°C.

The other method of purifying the crude product is to treat a dilute solution (2 to 3 per cent) of the impure *p*-chlorophenaceturic acid with urease. After standing overnight the urea is split off, and it is then only necessary to filter the hot solution, concentrate it on the water bath, and acidify it. One thus obtains fairly pure *p*-chlorophenaceturic acid. After two recrystallizations from hot water this melts at 164–165°C. The analysis for nitrogen according to the method of Kjeldahl gave the following results.

Found. 6.30 and 6.19 per cent.
Theoretical. 6.15 per cent.

Synthesis of p-Chlorophenaceturic Acid.

p-Chlorophenylacetyl chloride was made by the action of phosphorus pentachloride on *p*-chlorophenylacetic acid according to the method of Friedmann and Maase (4). *p*-Chlorophenaceturic acid was then prepared from this product by the interaction of the latter with glycocoll according to the method of Schotten and Baumann. The yield of *p*-chlorophenaceturic acid varied between 65 and 75 per cent of the theoretical.

5 gm. of this synthetic *p*-chlorophenaceturic acid were dissolved in 1,290 cc. (a 24 hour collection) of normal human urine. The urine was then evaporated on the water bath, acidified with sulfuric acid, and extracted with ethyl acetate. 4.2 gm. of the original *p*-chlorophenaceturic acid were thus recovered. Thereupon, the individual who had voided this urine ingested 5 gm. of the *p*-chlorophenaceturic acid on each of 2 successive days. The urine was treated in every respect like the urine after the ingestion of *p*-chlorophenylacetic acid, and the same impure *p*-chlorophenaceturic acid (urea additive compound) was obtained. Of the 10 gm. of *p*-chlorophenaceturic acid ingested, only 6.1 gm. were recovered.

The *p*-chlorophenylacetic acid was next fed to a rabbit. A healthy male received a total of 3 gm., in doses of 1 gm. on the 1st, 3rd, and 5th days of the experimental period. The urine

was treated exactly as was the human and dog urine following the feeding of the same compound. We were surprised to find only the uncombined acid in the urinary extract. After feeding the 3 gm., a total of approximately 2 gm. of the unchanged substance was recovered. Thinking that perhaps the injection of the material might lead to different results, 0.5 gm. of the substance in the form of a solution of its sodium salt was injected on each of 3 successive days. Again, however, only the uncombined acid could be recovered from the urine.

p-Bromophenylacetic Acid.

p-Bromophenylacetic acid was prepared from *p*-aminophenylacetic acid by the Friedl and Craft reaction. The product used in the experimental work melted at 116°C.

Three rabbits were fed 0.5 gm. apiece, as a water solution of the sodium salt, on each of 3 successive days until a total of 4.5 gm. had been given. The urine was collected, evaporated, acidified, and extracted with ethyl acetate as previously described. The ethyl acetate extract was evaporated nearly to dryness, 200 cc. of water were added, the remainder of the ethyl acetate was evaporated, and the water solution boiled with animal charcoal and filtered while hot. On cooling, about 2 gm. of the free *p*-bromophenylacetic acid crystallized out. This was filtered off, recrystallized, and dried. It then melted at 115–116°C. Concentration of the mother liquor of these crystals yielded another small amount of *p*-bromophenylacetic acid. No conjugation product of this compound was detected.

A large dog weighing 26.3 kilos was given *via stomach tube* a dose of 3 gm. of *p*-bromophenylacetic acid, and 2 days later received a second dose of 4 gm. The urine was collected for 48 hours after the second dose. After a week of rest this process was repeated, so that the dog received a total of 14 gm. of the acid. The urine was evaporated, and extracted in two portions, in the way described above. After extracting with ethyl acetate for a period of 9 hours the extracts were united, the ethyl acetate was distilled off, the residue taken up with 600 cc. of water, boiled with charcoal, and allowed to cool. Before the solution had entirely cooled, a heavy precipitate of yellow, granular crystals appeared. These were filtered off and recrystallized

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twice from hot water. After drying *in vacuo* the substance melted at 159–160°C. An analysis for nitrogen according to Kjeldahl gave the following results, indicating the presence of *p*-bromophenaceturic acid.

Found. 5.20 and 5.22 per cent.
Theoretical. 5.15 per cent.

A total of 8.7 gm. of the *p*-bromophenaceturic acid was thus isolated from the urine after the feeding of 14 gm. of *p*-bromophenylacetic acid. A trifle less than 2 gm. of the original acid was also recovered.

A man weighing 60 kilos ingested during the course of 6 days a total of 8 gm. of *p*-bromophenylacetic acid. From the urine 3.3 gm. of *p*-bromophenaceturic acid were extracted, together with somewhat less than 0.5 gm. of the original material ingested. The *p*-bromophenaceturic acid after recrystallization melted at 158–160°C.

Synthesis of p-Bromophenaceturic Acid.

p-Bromophenylacetyl chloride was prepared (a) by the action of phosphorus pentachloride on *p*-bromophenylacetic acid, and (b) using thionyl chloride instead of phosphorus pentachloride.

(a). 5 gm. of dry *p*-bromophenylacetic acid (m.p. 114–115°C.) were mixed with 4.9 gm. of dry phosphorus pentachloride in a Claisen flask. The reaction began immediately, with the formation of a liquid. After heating for a few minutes on a water bath, the phosphorus oxychloride was distilled off *in vacuo*. The *p*-bromophenylacetyl chloride was then distilled off under greatly reduced pressure (2 mm. of pressure, with the temperature of the oil bath at 160–170°C.). The yield of the acid chloride (b.p. 113°C.) was 2.4 gm., or 44 per cent of the theoretical.

(b). 5 gm. of the dry *p*-bromophenylacetic acid were added to 25 gm. of thionyl chloride and heated in a round bottom flask. This flask was then fitted with a reflux condenser and heated in an oil bath at about 100°C. until all the substance was in solution. The excess thionyl chloride was then distilled off *in vacuo*. The *p*-bromophenylacetyl chloride was then distilled off under reduced pressure (3 mm. of pressure, with the tempera-

ture of the oil bath at 180–190°C.). The product boiled at 118°C. The yield was 4.4 gm., or 81 per cent of the theoretical.

Analysis of the product obtained by the PCl_5 method.

Found.	15.05	per cent	chlorine.
Theoretical.	15.23	“ “ “	

Analysis of the product obtained by the SOCl_2 method.

Found.	14.97	per cent	chlorine.
Theoretical,	15.23	“ “ “	

The *p*-bromophenylacetyl chloride is a pale yellow liquid with a very penetrating odor. It is soluble in alcohol, ether, and benzene.

1.3 gm. of glycocoll were dissolved in 6.5 cc. of water. The solution was cooled and made alkaline with normal sodium hydroxide. 4 gm. of *p*-bromophenylacetyl chloride were then added drop by drop, with vigorous shaking and cooling. Sufficient normal sodium hydroxide was added from time to time to keep the solution slightly alkaline. After all the acid chloride had been added the contents of the flask were acidified to Congo red with sulfuric acid. The white precipitate was filtered off, thoroughly washed with ether, and dried. The mother liquor was also shaken repeatedly with ether to remove the free *p*-bromophenylacetic acid. *p*-Bromophenaceturic acid was found in both the precipitate and the mother liquor. The yield was 1.2 gm., or 38 per cent of the theoretical amount. It melted at 160–161°C. The substance is insoluble in cold water, ether, and benzene, but dissolves easily in hot water, ethyl acetate, alcohol, and petroleum ether. Analysis for nitrogen according to Kjeldahl resulted as follows:

Found.	5.03, 5.16, and 5.25	per cent.
Theoretical.	5.14	per cent.

Analysis for bromine according to Carius gave:

Found.	29.70	per cent.
Theoretical.	29.41	“ “

The *p*-bromophenaceturic acid was also prepared according to the method of Curtius, in order to determine the better method

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for the preparation of these substituted phenacetic acids. 2 gm. of *p*-bromophenylacetyl chloride and 1.2 gm. of the dry hydrochloride of the ethyl ester of glycocholic acid were placed in a round bottom flask. 10 cc. of dry benzene were then added, and the flask was heated on a water bath under a reflux condenser for 6 hours. The liquid was then filtered and evaporated by means of a current of warm air. Thereupon the ethyl ester of *p*-bromophenacetic acid crystallized out. After recrystallization from alcohol it melted at 106°C. Yield, 1.5 gm., or 58 per cent of the theoretical. The crystals, in the form of white leaflets, were insoluble in cold water and petroleum ether, but dissolved easily in hot water, alcohol, ether, ethyl acetate, and benzene. Analysis for nitrogen according to Kjeldahl yielded:

Found. 4.71, 4.73, and 4.77 per cent.
Theoretical. 4.67 per cent.

1 gm. of *p*-bromophenacetic acid-ethyl ester was dissolved in 10 per cent sodium hydroxide by gentle heating on a water bath. Upon acidification with dilute sulfuric acid the free *p*-bromophenacetic acid crystallized out. After recrystallization from water it melted at 160–161°C. Yield, 0.6 gm., or 47 per cent.

p-Bromophenacetic Acid Nitrile.

5 gm. of aminoacetonitrile were dissolved in 8.3 cc. of water and the solution was cooled in an ice bath. Then 4.66 gm. of *p*-bromophenylacetyl chloride were dissolved in 8.3 cc. of benzene and added drop by drop to the solution of the nitrile, which had been made alkaline with normal sodium hydroxide. The container was shaken vigorously and cooled after each addition. The solution was also kept alkaline by the addition from time to time of small amounts of sodium hydroxide. The nitrile of *p*-bromophenacetic acid precipitated out, was filtered off, and washed with cold water. After recrystallization from hot water it melted at 137°C. Yield, 3.4 gm., or 68 per cent. The substance crystallizes from hot water as long, shiny, white prisms, insoluble in cold water, ether, benzene, and petroleum ether, but

soluble in hot water, alcohol, and ethyl acetate. Analysis for bromine according to Carius resulted as follows:

Found.	31.34	per cent.
Theoretical.	31.62	" "

Silver Salt of p-Bromophenaceturic Acid.

1 gm. of *p*-bromophenaceturic acid was dissolved in dilute ammonium hydroxide. Dilute nitric acid was then added to neutrality. 0.6 gm. of silver nitrate was dissolved in a small amount of water and added with stirring to the neutral solution of the *p*-bromophenaceturic acid. The silver salt of the *p*-bromophenaceturic acid precipitated out as a thick, white mass. This was filtered off, washed well with cold water, and recrystallized from hot water. Yield, 1.3 gm., or 92 per cent. Analysis of the substance for silver showed the following results.

Found.	28.17	per cent.
Theoretical.	28.31	" "

Ethyl Ester of p-Bromophenaceturic Acid.

The ethyl ester of *p*-bromophenaceturic acid was made from the silver salt as follows: 0.5 gm. of the silver salt was treated with just enough alcohol to form a thick paste. To this paste 0.2 gm. of ethyl iodide was added. The mixture was then gently heated on a water bath, and the silver iodide was filtered off. Upon evaporation of alcohol, the ethyl ester of *p*-bromophenaceturic acid is obtained (m.p. 106°C.). Yield, 0.05 gm., or 14 per cent of the theoretical.

p-Aminophenylacetic Acid.

p-Aminophenylacetic acid was prepared by the reduction of *p*-nitrophenylacetic acid according to the method of Jacobs and Heidelberger (8). After two recrystallizations from hot water it melted at 200–202°C.

A large dog of 22 kilos body weight was fed 1 gm. of the acid on each of 5 consecutive days. The urine was evaporated and extracted with ethyl acetate. The only substance that could be found in the water solution of the ethyl acetate residue was

traces of the free amino acid. It was only after large doses of 5 to 7 gm. of the acid had been fed to the dog that any positive results were obtained. The animal thus received a total of 25 gm. of the substance in four doses of 5, 6, 7, and 7 gm., respectively, on 4 consecutive days. The dog apparently suffered no discomfort as he ate and drank normally during the entire experimental period.

From earlier work done by Salkowski (9), as well as by Hildebrandt (10), on *m*- and *p*-aminobenzoic acids when experimenting with dogs, we were led to expect either the excretion of the free acid or the formation of a uramino acid. If the former course should prove to be the route taken by our *p*-aminophenylacetic acid, then it was evident that the urine should not be acidified with hydrochloric or sulfuric acid on account of the ease with which these mineral acids form readily soluble products with the amino compounds. Hence after evaporating the urine we acidified it strongly with *o*-phosphoric acid. We then extracted it repeatedly with ethyl acetate in a continuous extracting apparatus. The residue from the united extracts was taken up with hot water and boiled with charcoal, resulting in 500 cc. of a straw-colored solution. As no crystals appeared on cooling, the solution was gradually evaporated to 200 cc., at which point an amorphous precipitate appeared. The solution was then diluted to 1,500 cc., filtered hot, then suddenly cooled in an ice-salt bath, while the sides of the container were being rubbed vigorously with a glass rod. There formed a very copious precipitate of fine, white, granular crystals on the sides and bottom of the beaker. This was followed by a precipitate of light yellow, crystalline leaves. The substance, when dry, proved to be impure, as it started to melt at about 170°C. and continued up to 210°C. The nitrogen determinations also showed the presence of two or more substances. The only method of separation seemed to be by extracting with ether, as the one substance, found to be free *p*-aminophenylacetic acid, was considerably more soluble in ether than was the other substance,—*p*-aminophenaceturic acid. Unfortunately, while *p*-aminophenylacetic acid melts quite sharply at 200–201°C., *p*-aminophenaceturic acid also sinters and practically decomposes at the same temperature. This also agrees with the observations of Hotter (7)

who made some of this latter compound synthetically in an attempt to synthesize *p*-hydroxyphenaceturic acid. After thus separating the *p*-aminophenylacetic acid and recrystallizing it several times from hot water it melted sharply at 200°C., and gave the following analysis for nitrogen according to the Kjeldahl method.

Found. 9.30 and 9.19 per cent.

Theoretical. 9.27 per cent.

The water solution remaining after the extraction with ether was concentrated. A sandy, crystalline residue formed on the bottom of the beaker. This proved to be *p*-aminophenaceturic acid, and showed the following properties after repeated recrystallizations from water: sinters at 200–204°C.; forms soluble additive compounds with mineral acids, showing the presence of a free amino group; forms, on hydrolysis, *p*-aminophenylacetic acid and glycocoll. Analysis for nitrogen according to Kjeldahl gave the following results.

Found. 13.50 and 13.60 per cent.

Theoretical. 13.46 per cent.

Combustion of the material yielded the following figures for carbon and hydrogen.

Found. C 57.66, H 5.90 per cent.

Theoretical. " 57.69, " 5.77 " "

Synthesis of p-Aminophenaceturic Acid.

p-Aminophenaceturic acid was synthesized according to the following method. Phenaceturic acid itself was prepared from phenylacetyl chloride and glycocoll according to the method of Schotten and Baumann. 5 gm. of phenaceturic acid (m.p. 143°C.) were then treated with 20 gm. of concentrated nitric acid and 30 gm. of concentrated sulfuric acid. The mixture was placed in an ice bath and kept at 0°C. for an hour. At the end of this time the contents of the flask were poured onto cracked ice and allowed to stand in the ice box overnight. A yield of 4.7 gm. of *p*-nitrophenaceturic acid, melting at 166–169°C., was thus obtained. After one recrystallization from water the melting point rose to 169–170°C. This *p*-nitrophenaceturic

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acid was then converted into its barium salt and reduced by means of ferrous sulfate and ammonia. A rather poor yield of *p*-aminophenaceturic acid was thus obtained, which sintered at 200°C.

A man ingested 20 gm. of *p*-aminophenylacetic acid in the course of 8 days, taking a dose of 5 gm. every other day. The urine was collected and extracted with ethyl acetate. The residue was then taken up in hot water and boiled with charcoal. A substance was thus obtained which, after recrystallization from hot water, melted at 157°C. This substance was recrystallized ten times from water and finally gave a constant and sharp melting point of 167°C. It then proved to be *p*-acetylaminophenylacetic acid, as the following analytical data indicate. Analysis for nitrogen according to the method of Dumas:

Found.	7.44	per cent.
Theoretical.	7.26	" "

Analysis for carbon and hydrogen by combustion:

Found.	C	62.37,	H	6.05	per cent.
Theoretical.	"	62.18,	"	5.69	" "

For further identification, 1 gm. of the substance was dissolved in water, and bromine water was added until the solution was no longer decolorized. This solution was then allowed to stand in the ice box overnight. In the morning small, wart-like crystals appeared on the bottom of the beaker. These were filtered off and dried in an exiccator. They were then found to melt at 162°C.,—the m.p. of *m*-bromo-*p*-acetylaminophenylacetic acid,—and gave the following analytical figures.

Found.	N	5.14,	C	44.12,	H	3.67	per cent.
Theoretical.	"	5.37,	"	43.97,	"	3.85	" "

The melting point of the *p*-acetylaminophenylacetic acid corresponded with that of the same substance made synthetically by the interaction of acetic anhydride and *p*-aminophenylacetic acid.

1 gm. of *p*-aminophenylacetic acid was fed to a rabbit daily for 5 days. The urine was found to contain about 20 per cent of the material, and this acetylated. Very small quantities of the free acid were also found, but no conjugation product with glyco-coll was in evidence.

p-Acetylaminophenylacetic Acid.

This substance was prepared in the laboratory and fed to a dog to determine its toxicity as well as to see whether it would be further detoxicated by being combined with glycocoll to form *p*-acetylaminophenacetic acid. It was found, however, that the material was not at all toxic to the dog, as an animal of 11.8 kilos body weight showed no signs of discomfort after a dose of 3 gm. of the substance. Moreover, 72 per cent of the ingested material was excreted unchanged.

DISCUSSION.

In the detoxication both of *p*-bromophenylacetic acid and of *p*-chlorophenylacetic acid the human organism employs the same reaction as does the dog; that is, by conjugating the substances with glycocoll and excreting them as the corresponding halogenated phenacetic acids. The human reaction, however, differs slightly inasmuch as it forms a small amount of an additive compound of urea with *p*-chlorophenacetic acid. Moreover, it is seen that the *p* substitution products of phenylacetic acid, like the *o* substitution products (2), are detoxicated by glycocoll instead of by glutamine (1).

The rabbit differs from both the human being and the dog in detoxicating these two halogen derivatives of phenylacetic acid inasmuch as he excretes them uncombined.

The fact that *p*-aminophenylacetic acid is acetylated by the human being and by the rabbit, while the dog allows the amino group to remain free and detoxicates the compound by joining it through the —COOH group to glycocoll, is noteworthy.

In the literature one finds various cases where the dog is able to acetylate amino groups of an aliphatic acid, or similar groups in the side chain of an aromatic acid. This reaction, however, is much rarer for the rabbit and for man. In the case before us, where the amino group is bound directly to the ring, acetylation is made use of by man and by the rabbit, but not by the dog. We are studying this reaction further.

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STUDIES OF AUTOLYSIS.

XIII. THE KINETICS OF THE AUTOLYTIC MECHANISM.

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Sufficient evidence has accumulated to show that autolysis takes place in two stages. The native cell proteins are broken down to smaller fragments through the action of primary proteases in an acid medium. These fragments in turn are split to amino acids by ereptic-like enzymes of the cells (1, 2).

In the present paper we wish to present data bearing upon the equilibria and kinetics of these two phases of the reaction, and upon the mechanism involved in the inhibition of autolysis by certain foreign proteins. Though the data are not complete, they are sufficient to warrant a preliminary attempt to formulate the kinetics of the autolytic phenomena in definite terms.

The material used throughout was liver. A study has been made of the relation between coagulable and non-coagulable nitrogenous material in the digestions, using trichloroacetic acid as the coagulant. The total amino acid nitrogen that might theoretically be considered available in the digests was determined by the Van Slyke method (3) after 24 hours hydrolysis on the water bath with 25 per cent HCl. Amino acids actually produced in autolysis were determined in the usual way by Sørensen titration (4). The amino N liberated was calculated, assuming the general

NH₂
|
formula R—C—COOH, with 1 gram atom of amino N for
 |
 H

every gram equivalent of amino acid. Total nitrogen was determined by the Kjeldahl-Gunning method, and all figures have been calculated for 25 cc. of the trichloroacetic acid filtrate, which

represents 6.25 cc. of the original liver brei made up in the way we have adopted as standard and described in previous papers of this series.

TABLE I.
Extent of the Primary Cleavage of Pig Liver.

No.	Condition.	Initial pH.	Length of auto-lysis.	Total N in digest.	Total N in filtrate.	Total N in non-coagu-lable state.
			days	mg.	mg.	per cent
1	Control.	4.0	18	41.6	40.7	97.8
2	" + egg albumin.	4.0	18	48.4	47.3	97.8
3	"	4.5	27	18.6	19.15	102.9
4	"	4.5	10	20.5	19.05	92.8
5	"	6.0	10	20.5	14.0	69.3
6	"	6.5	27	18.9	9.7	51.3
7	"	6.8	150	42.3	21.6	51.1
8	" + 25 cc. egg white.	6.8	150	52.3	21.15	40.4
9	"	4.0	2	37.45	29.45	78.6
10	" + egg white.	4.0	2	54.0	41.4	76.6

TABLE II.
Velocity of Primary Cleavage of Pig Liver.

No.	Condition.	Initial pH.	Total N in digest.	Time in days.					
				0		2		15	
				Non-coagulable N.	Percentage of total N.	Non-coagulable N.	Percentage of total N.	Non-coagulable N.	Percentage of total N.
				mg.	mg.	mg.	mg.	mg.	mg.
1	Control.	6.7	37.45	5.6	14.95	15.27	40.3	18.63	49.7
2	"	4.0	37.45	5.6	14.95	29.45	78.6	35.1	93.7
3	" + 50 cc. egg white.	6.7	54.0	5.88	10.88	8.82	16.35	11.0	20.35
4	Control + 50 cc. egg white.	4.0	54.0	5.88	10.88	41.4	76.6	56.2	104.0

Primary Cleavage.

The extent of primary cleavage has been determined as the fraction of total nitrogen which appears in the non-coagulable

state. This figure is undoubtedly too low during the early hours of autolysis, since trichloroacetic acid precipitates some of the larger protein fragments such as the proteose group. It nevertheless gives an approximation which becomes more accurate as digestion proceeds with the further cleavage of these larger initial cleavage products. (See Table I.)

The data show that at the optimum pH (4.5) cleavage to the non-coagulable state is complete. Even in 2 days it is nearly 80 per cent complete. At lower H ion concentrations (pH 6.0 to 6.7) it is less complete, but still above 50 per cent.

The velocity of primary cleavage is indicated by the data in Table II.

In the acid digests primary cleavage is nearly complete in 2 days. Prolonged autolysis effects a comparatively small increase in non-coagulable nitrogen, and this increase probably represents secondary cleavage of the complex proteoses, rather than primary cleavage. Primary cleavage therefore is even more rapid than our data would indicate.

At the end of 15 days primary cleavage is practically complete, and the amount of non-coagulable nitrogen at that time is an index of the total substratum available. At a given time, for example 2 days, primary cleavage is a constant fraction of the total available substratum, independent of the H ion concentration. Thus at pH 6.7 cleavage of available substratum is 82 per cent complete, while at pH 4.0 cleavage is 84 per cent complete. The relative velocities of the two reactions are the same regardless of H ion concentration, so that there is no clear evidence of increased activity of the primary protease at the higher H ion level, and the effect of acid appears to be merely that of increasing substratum.

At optimum pH there is no evidence of equilibrium between native protein and primary cleavage products. Whether or not an equilibrium exists at lower H ion concentrations cannot be stated at present.

Secondary Cleavage.

The percentage of total available amino nitrogen appearing as amino acid in the filtrates has been calculated. The results of such calculations show that in no case is cleavage to the amino

acid stage complete. Representative examples are given in Table III.

Even under optimum conditions only one-third of the total available amino nitrogen appears as amino acid. Under these conditions the primary cleavage is complete. At lower acidities primary cleavage is incomplete, but always more complete than the secondary. This difference is in part due to differences in total amino nitrogen and total nitrogen in the digests. Thus a digest at pH 6.5 contained 15.4 mg. of total available amino nitrogen and 18.9 mg. of total nitrogen, a difference of 22.7 per cent. The amino acid liberated was 24.0 per cent of the total available,

TABLE III.
Extent of Cleavage to Amino Acid Stage of Pig Liver.

No.	Condition.	Initial pH.	Length of autolysis.	Total available amino N.	Amino N liberated.	Percentage of total available amino N.
			<i>days</i>	<i>mg.</i>	<i>mg.</i>	
1	Control.	6.5	27	15.4	3.7	24.0
2	"	6.0	27	15.4	4.6	30.0
3	"	5.5	27	15.4	5.32	34.5
4	"	5.0	27	15.4	6.0	39.2
5	"	4.5	27	15.4	6.58	42.7
6	"	4.0	27	15.4	6.6	42.7
7	"	3.5	27	15.4	5.9	38.2
8	"	5.5	18	39.1	10.8	27.6
9	"	4.5	19	39.1	14.6	37.2
10	"	4.5	10	16.35	7.0	42.8

while digestion to non-coagulable nitrogen was 51.3 per cent of the total, a difference of 27.3 per cent. At the optimum pH, however, this explanation does not apply.

These results indicate an equilibrium in an autolyzing brei between amino and peptide nitrogen. If amino nitrogen is calculated in terms of percentage of non-coagulable nitrogen, a strikingly constant value is found, regardless of conditions obtaining in the digest. (See Table IV.)

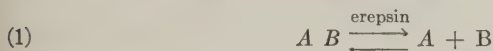
The average of eighteen such determinations is 37.7 per cent, and the variations from the average bear no constant relation to H ion concentration or to the presence of foreign protein or peptone. A certain fraction of the primary cleavage products is

broken down to amino acid. This indicates that the secondary cleavage reaction reaches an equilibrium determined by the concentrations of substrata and products. If the mass of substratum is increased, either by increased primary cleavage or addition of peptone, the amino acid titer will be correspondingly increased,

TABLE IV.
Percentage of Non-Coagulable N as Amino N in Pig Liver.

No.	Condition.	Initial pH.	Length of auto-lysis.	Non-coagu- lable N.	Amino N.	Per- centage of non-coagu- lable N.
			<i>days</i>	<i>mg.</i>	<i>mg.</i>	
1	Control.	4.0	18	40.7	15.1	37.1
2	" + egg albumin.	4.0	18	47.3	17.1	36.15
3	"	6.5	27	9.7	3.7	38.15
4	"	4.5	27	19.15	6.58	34.25
5	"	6.0	10	14.0	4.82	34.4
6	"	4.5	10	19.05	7.00	36.8
7	"	6.78	150	21.6	9.4	43.5
8	" + 25 cc. egg white.	6.78	150	21.15	8.2	38.8
9	" + 50 " " "	6.78	150	18.75	7.45	39.7
10	" + 100 " " "	6.78	150	18.50	6.22	33.6
11	"	6.7	120	17.32	7.42	42.8
12	" + 50 cc. egg white.	6.7	120	10.6	4.25	40.0
13	" + 50 " peptone.	6.7	120	30.25	12.5	40.9
14	" + 50 " egg white + 50 cc. peptone.	6.7	120	29.4	11.3	38.4
15	Control.	6.7	15	18.63	8.26	44.4
16	" + 50 cc. egg white.	6.7	15	11.0	3.89	35.35
17	"	4.0	15	35.1	11.35	32.35
18	" + 50 cc. egg white.	4.0	15	56.2	18.75	33.4
Average.....						37.7

but only the same proportion of the substratum will be broken up. We may represent this reaction by the equation



The equilibrium point is independent of the H ion concentration within the range studied though the velocity of the reaction in both directions is very probably influenced by the H ion concentration. The kinetics of this reaction may be studied in the

following manner. The velocity of the direct reaction may be expressed by

$$(2) \quad \frac{dx}{dt} = k(a - x)$$

where k is the velocity constant, dx the small increase in the amount of x , during the small interval of time dt , and a is the initial concentration of AB . Similarly, the reverse reaction is represented by

$$(3) \quad \frac{dx'}{dt} = k' (b + x) (c + x)$$

where b is the initial concentration of A and c the initial concentration of B . Then the total velocity due to the opposing reactions will be

$$(4) \quad \frac{dX}{dt} = \frac{dx}{dt} - \frac{dx'}{dt}$$

At equilibrium, when $\frac{dX}{dt} = 0$, the equilibrium constant is obtained from

$$(5) \quad K = \frac{k}{k'} = \frac{(b + x) (c + x)}{(a - x)}$$

In order to apply equation (5) to the actual reaction, it is necessary to make certain assumptions. In the first place, the concentration of AB is in reality the concentration of the primary and secondary cleavage products of the proteins; namely, proteoses and peptones. The higher cleavage products are coagulable by trichloroacetic acid and, therefore, will not appear in the total nitrogen of the filtrate. On the other hand, the total nitrogen of the digests is not entirely amino nitrogen though a large percentage is. These two factors will tend to balance each other though it is not known to what extent this takes place. In the second place, the concentration of AB is being influenced simultaneously by two factors. It is increased by the primary cleavage of proteins and decreased by the action of erepsin. However, at the time of equilibrium, the primary cleavage is complete. Therefore, we have used the non-coagulable N of the filtrates at the end of autolysis as an index of the initial concentration of AB .

The primary cleavage is so nearly complete in the 1st or 2nd day that no great error is introduced.

The concentration of *A* and *B* is represented by the amino N figures. In reality, *A* and *B* represent a variety of amino acids, but for our purpose, these may be considered as a single

TABLE V.
Equilibrium Constants.

No.	Condition.	Initial pH.	<i>b</i>	<i>x</i>	<i>a</i>	<i>K</i>
1	Control.	4.0	1.12	14.0	40.7	0.557
2	" + albumin.	4.0	1.12	15.97	47.3	0.546
3	"	6.5	0.98	2.72	9.7	0.53
4	"	4.5	0.98	5.6	19.15	0.496
5	"	6.0	1.215	3.61	14.0	0.464
6	"	4.5	1.215	5.87	19.05	0.537
7	"	6.78	1.12	8.17	21.6	0.747
8	" + albumin.	6.78	1.12	7.08	21.15	0.627
9	" + "	6.78	1.12	6.33	18.75	0.600
10	" + "	6.78	1.12	5.10	18.5	0.464
11	"	6.7	1.12	6.30	17.32	0.672
12	" + albumin.	6.7	1.12	3.135	10.6	0.57
13	" + peptone.	6.7	3.23	11.43	30.25	0.778
14	" + " + albumin.	6.7	3.23	10.17	29.4	0.697
15	Control.	6.7	1.4	6.86	18.63	0.701
16	" + albumin.	6.7	1.4	2.49	11.0	0.457
17	"	4.0	1.4	9.94	35.1	0.451
18	" + albumin.	4.0	1.4	17.37	56.2	0.473
Average.....						0.564

substance and consequently the equation for the reverse reaction reduces to

$$(6) \quad \frac{dx'}{dt} = k' (b - x)$$

Substituting in equation (5), we have,

$$(7) \quad K = \frac{(b - x)}{(a - x)}$$

In equation (7), *K* represents the equilibrium constant, *b* the initial concentration of amino acid nitrogen, *x* the increase in

amino acid nitrogen, and *a* the non-coagulable nitrogen at the end of primary cleavage. The results of a series of calculations for a variety of conditions are given in Table V.

The average value for the equilibrium constant is 0.56 with a maximum variation of 0.214. The variations from the average are apparently at random and bear no relation to the H ion concentration or other conditions. The essential correctness of this method of calculating the equilibrium constant is indicated by the results obtained on calculating the non-coagulable nitrogen

TABLE VI.
Non-Coagulable N, Observed and Calculated.

No.	Condition.	Approximate pH.	<i>b</i>	α	<i>a</i>	N observed.	N calculated.
1	Control.	6.5	0.70	1.54		6.85	5.5
2	" + HCl.	4.0	0.84	8.13		24.1	24.0

TABLE VII.
Inhibitory Effect of Egg White on Autolysis as Related to the Amount of Egg White.

No.	Condition.	Initial pH.	0.2 N amino acid.						
			Days.				Months.		
			0	1	5	11	1	4	5
			cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	Control.	6.78	0.40	1.55	2.02	2.35	2.65	3.28	3.32
2	“ + 25 cc. egg white.	6.78	0.40	1.20	1.35	1.75	2.27	2.89	2.93
3	“ + 50 “ “ “	6.78	0.40	0.96	1.24	1.50	1.75	2.43	2.66
4	“ + 100 “ “ “	6.78	0.40	0.94	1.17	1.33	1.63	2.08	2.22
5	“ + 150 “ “ “	6.78	0.40	0.77	0.97	1.10	1.26	1.48	

for certain autolyzing digests described by Bradley and Taylor (5). These results are given in Table VI and show close agreement between the calculated and observed values for the non-coagulable nitrogen.

Inhibition of Autolysis.

In a preceding paper we noted the inhibitory effect of certain proteins on liver autolysis and suggested that the inhibition was due to combination between protein and enzyme, resulting in the removal of enzyme from the field of action. Certain experiments have been designed to test this hypothesis.

If inhibition is due to the formation of a stable protein-enzyme complex, the amount of inhibition should be related to the amount of protein added. The results show this to be the case, inhibition increasing with increasing amounts of egg white. (See Table VII.)

The question arises, what protease in the liver brei is involved in the inhibitory effect? Since the amount of autolysis is determined primarily by the amount of primary cleavage, inhibition of the primary protease would be revealed in both the amino acid and non-coagulable nitrogen figures. Decrease in amino acid

TABLE VIII.

Effect of Egg White on the Cleavage of Peptone by the Liver Enzymes.

No.	Condition.	Initial pH.	0.2 N amino acid.					Non-coagulable N.	Amino N.	Percentage of non-coagulable N appearing as amino N.
			0 day.	1 day.	8 days.	4 mos.	Difference.			
1	Control.	6.7	0.40	1.06	1.71	2.65	1.13	17.32	7.42	42.8
2	" + 50 cc. egg white.	6.7	0.40	0.80	1.15	1.52		10.6	4.25	40.0
3	Control + 50 cc. peptone.	6.7	1.07	3.07	3.92	5.15	0.45	30.25	12.5	40.9
4	Control + 50 cc. egg white + 50 cc. peptone.	6.7	1.07	2.80	3.52	4.70		29.4	11.3	38.4

titer without corresponding decrease in non-coagulable nitrogen would indicate that the primary protease is unaffected while erepsin is inhibited. The figures in Table VIII show that the primary protease is the enzyme inhibited and that erepsin is unaffected since it is free to act on peptone in the presence of egg white. This conclusion is further borne out by the fact that a constant fraction of non-coagulable nitrogen is present as amino nitrogen whether proteolysis as a whole has been increased or decreased in amount. This can only mean, as has been shown above, that the cleavage of proteoses and peptones by erepsin is governed by the laws of mass action and is not inhibited by the presence of the foreign protein.

Having shown that in all probability it is the primary protease that is inhibited, experiments were devised to test the hypothesis that the inhibition was due to the formation of a stable protein-enzyme complex. According to our conception, the stability of this complex (which in turn determines whether proteolysis shall occur or not) is determined by the H ion concentration. Con-

TABLE IX.

Effect of Acidification on the Inhibition of Autolysis by Egg White.

No.	Condition.	Initial pH.	0.2 N amino acid.			
			0 day.	2 days.	15 days.	15 days.*
1	Control.	6.7	0.50	2.43	2.95	3.17
2	" + 50 cc. egg white.	6.7	0.50	1.17	1.39	2.02
3	"	4.0	0.50	2.84	4.05	
4	" + 50 cc. egg white.	4.0	0.50	2.97	6.70	

* Acidified on 1st day; 14 days subsequent autolysis.

TABLE X.

Non-Coagulable N for Table IX.

No.	Total N.	Time in days.							
		0		2		15		15*	
		N	Per-centage of total N.	N	Per-centage of total N.	N	Per-centage of total N.	N	Per-centage of total N.
	mg.	mg.		mg.		mg.		mg.	
1	37.45	5.60	14.95	15.27	40.3	18.63	49.7	28.15	75.0
2	54.0	5.88	10.88	8.82	16.35	11.0	20.35	40.3	74.7
3	37.45	5.60	14.95	29.45	78.6	35.1	93.7		
4	54.0	5.88	10.88	41.4	76.6	56.2	104.0		

* Acidified on 1st day; 14 days subsequent autolysis.

sequently, if the latter is increased below the isoelectric point of the protein, inhibition does not occur as has been shown in the preceding paper. Further, the complex formed may or may not be reversible. If reversible, a digest in which inhibition is apparent should later show active proteolysis if acidified to an H ion concentration above the isoelectric point of the protein. In Tables IX and X are given the results of such an experiment.

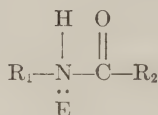
From the amino acid titer, one would conclude that the inhibition is reversible only to a slight extent. The non-coagulable nitrogen figures are illuminating, however. A marked increase in proteolysis occurs in both the control and control plus egg white, showing that the primary protease-protein combination is one whose stability is determined by the H ion concentration. The larger amount of non-coagulable nitrogen in the control plus egg white emphasizes this. The difference in the liberation of non-coagulable nitrogen and amino nitrogen is probably due to changes in the activity of erepsin. The values for the equilibrium constant are 0.427 and 0.163 for the control and control plus egg white (after acidification), respectively. These low values show that the activity of erepsin has been altered by some unknown factor, perhaps rapid destruction of the enzyme.

DISCUSSION.

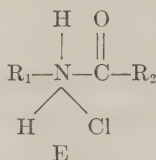
In autolysis, the first step in the process is believed to be combination between the primary protease and the native proteins of the tissue cells. During life and the normal pH of the cell, the protease probably exists in this combined form. At the pH of 7.4 most of the tissue proteins are in the form of their base salts. This form is stable and no cleavage results. When, however, acid develops in the mixture acid proteins are formed, unstable in combination with the enzyme and with a weakened peptide bond. Under these conditions cleavage follows. Foreign base proteins added to a liver brei combine with a fraction of the protease and as the combination is stable, this fraction of the enzyme is removed from the field of the reaction. The speed of cleavage is thus retarded by adding base protein and in proportion to the amount added. Proteins with isoelectric points well on the acid side of neutrality produce the most marked retardation of autolysis. They remain base protein salts even when the liver proteins have largely been transformed into acid salts. Thus egg albumin and serum globulin with isoelectric points of pH 4.8 and 4.4, respectively, produce strong inhibition, while edestin with an isoelectric point of pH 6.9 does not. The retarding effect of a foreign protein disappears when it is converted into its acid salt, and at the same time it becomes substratum itself. It seems clear, therefore, that the cleavage of the native proteins in the

autolytic process is conditioned by the instability of the peptide linkage under the combined effect of acid and protease.

It is probable that the enzyme is attached to the nitrogen atom, and that the stability of such a combination depends on the amount of potential valence available. When the protein is in the form of base protein salt we may indicate the enzyme fixation thus:



This configuration is stable. Nitrogen is trivalent in its combination and the enzyme strongly attached. When the acid salt is formed, the nitrogen is presumably pentavalent, with no potential valencies left for fixation of the enzyme, thus:



This unstable configuration then tends to break up hydrolytically and also to free the enzyme. Satisfying the valences of nitrogen appears to weaken the bond to the carbon as well as the enzyme attachment. Cleavage of the native protein thus takes place at a few points giving rise to a small number of proteose fragments which are then subject to secondary disintegration.

Secondary cleavage is effected by the creptic enzyme of the cell. Acidity is not a necessary condition for the disintegration of proteose and peptone molecules, nor is this enzyme fixed by native proteins such as egg albumin. The presence of albumin in a liver brei does not retard cleavage of peptone to amino acid.

Secondary cleavage is never complete in autolysis *in vitro*. An equilibrium is attained when a constant fraction of the substratum has been converted into amino acid. The value of this fraction is apparently independent of the factors which influence primary cleavage, such as the H ion concentration. Liver

erepsin, therefore, appears to behave as a true catalyst, influencing velocity but not final equilibrium in the reaction which it facilitates.

SUMMARY.

1. Primary cleavage of the substratum, protein-acid-salt, is complete in autolysis, unless the primary protease is destroyed or inhibited.

2. Cleavage to the amino acid stage is incomplete; there is evidence of an equilibrium between amino acids and polypeptides.

3. The equilibrium constant for the action of erepsin has been determined, its value is 0.56. The equilibrium is independent of the H ion concentration within wide limits.

4. Inhibition of autolysis by a foreign protein is due to fixation of some of the primary protease.

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ON THE ACTION OF THYROXIN.

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Recently¹ Boothby and Sandiford recorded the observation that it is possible to construct plots integrating the increased rate of heat production for given weights of thyroxin. These authors pointed out that the curve of decay, when plotted upon semilogarithmic paper, tended to be a straight line. From these results they inferred that they were dealing with a monomolecular reaction and that the rate of excess heat production following the administration of thyroxin is dependent, directly, upon the concentration of the latter in the body tissues. In their brief communication detailed data are not given, but the writer assumes that they calculated the constants according to the formulation for the different orders and thus applied to their studies the differential equation

$$\frac{dx}{dt} = k(a - x)$$

in which a is the original quantity, x is the decrement in time t , and k is the coefficient of velocity, and have found a relation between the values of x and t .

Following the administration of thyroxin the general character of the calorigenesis-time curve is that of a gradual rise to a maximum, followed by a relatively much more gradual fall, the relative durations of rise and fall being approximately, 1:4; i.e., 10 days to 6 weeks. The hypothesis that the excess heat production is a function of the concentration of thyroxin in the tissues appears to be a plausible explanation of the nature of the latter part of the curve (period of decay). It does not, however, explain the nature of the earlier part of the curve; namely, the

¹ Boothby, W. M., and Sandiford, I., *J. Biol. Chem.*, 1924, lix, p. xl.

period during which the heat production is increasing. Since thyroxin once administered intravenously may be regarded as being in the tissues, it is difficult to understand why the maximum effect is so long in appearing.

The purpose of this communication is to record the data which have been obtained in an effort to explain the nature of the period of rise in the calorigenesis curve after dosage with thyroxin.

Since we are dealing with a reaction which increases in magnitude for a number of days after the administration of a single dose of drug, it is obvious that the concentration of that drug in the tissues is not the *only* governing factor. It occurs to the writer that the reaction may depend upon an increase in the amount of *active* protoplasmic mass. His conception is that under basal metabolism conditions only a minimum of protoplasmic mass is *active* and with an increase in metabolic rate more mass (reserve) becomes active. The increase continues until a maximum is reached which is dependent upon the concentration of thyroxin. This idea finds an analogy in other conditions. For example, the velocity of a given reaction is usually markedly influenced by alterations in temperature. Thus a rise of 10°C. frequently doubles the rate of reaction (van't Hoff). One explanation offered for this high temperature coefficient is that probably only a certain small proportion of the total number of molecules is active at any one temperature, this proportion increasing as the temperature rises. This analogy is particularly applicable here, since it has been found that, in the human body, the effect of changes in temperature (fever) upon the basal metabolism is somewhat similar to that noted in chemical reactions *in vitro*.

If this explanation be correct we would probably be dealing with a form of growth in which the rate of increment is at any particular instant proportional to the magnitude of that which is increasing. Thus, the successive rates of heat production at the end of a series of intervals would form the terms of a geometrical progression. This was tested by experiment.

Procedure.

The data for analysis were obtained as follows: The subjects were prepared according to the accepted method for basal metabolism studies (postabsorptive state, complete muscular rest, etc.).

The heat production (indirect method) per square meter per hour was determined, accepting the average of three determinations, all agreeing within the limits of experimental error. 10 mg. of thyroxin were then injected, intravenously, and the basal metabolic rate was determined daily for a period of about 10 days. The choice of the latter period was purely arbitrary, since that period is accepted as the average time required for thyroxin to attain its maximum effect.

The clinical data are only very briefly recorded, having no important bearing upon the problem under consideration. They are as shown in Table I.

TABLE I.

Case No.	Sex.	Age.	Diagnosis.	Basal meta- bolic rate.
		<i>yrs.</i>		<i>per cent</i>
1	Male.	38	Normal.	-5.3
2	"	43	Hypothyroidism(?).	-9.4
3	Female.	32	Obesity.	-3.6
4	Male.	26	Hypothyroidism(?).	-8.3
5	Female.	35	Obesity.	-5.9
6	Male.	49	Hypothyroidism.	-17.6

The most general form for the equation of such a progression is, in exponential form

$$Q_t = Q_0 e^{kt} \text{ or in logarithmic form } k = \frac{2.302}{t} \log_{10} \frac{Q_0 + X_t}{Q_0}$$

Where

Q_0 = original quantity.

k = constant.

Q_t = amount to which Q_0 has grown in time t .

x_t = increment during time t .

If the law is applicable it should be possible to predict the heat production at the end of any period of observation, once the constant of increment is known. For example:

Cal. per sq.m. per hr. before administration of thyroxin = 37.4

" " " " " 10 days after " " " = 45.1

$$\begin{aligned}
 k &= \frac{2.302}{10} \log_{10} \frac{37.4 + 7.7}{37.4} \\
 &= 0.2302 \times \log_{10} 1.205 \\
 &= 0.0186
 \end{aligned}$$

TABLE II.

Case No.	Day.	Cal. per sq. m. per hr.		Percentage variation.	<i>k</i>	Case No.	Day.	Cal. per sq. m. per hr.		Percentage variation.	<i>k</i>
		Actual.	Calculated.					Actual.	Calculated.		
1	Basal.	37.0				2	Basal.	34.9			
	1	39.41	38.02	+3.6	0.0624		1	37.14	36.16	+2.4	0.0621
	2	38.09	38.63	-1.4	0.0128		2	35.74	37.46	-4.6	0.0128
	3	38.72	39.27	-4.0	0.0149		3	38.95	38.82	+0.3	0.0366
	4	41.02	39.92	+2.7	0.0231		4	40.70	40.22	+1.1	0.0384
	5	41.21	40.58	+1.0	0.0194		5	41.19	41.68	-1.2	0.0331
	6	38.86	41.25	-5.8	0.0081		6	43.37	43.18	+0.4	0.0362
	7	43.00	41.93	+2.5	0.0193		7	44.8			
	8	44.39	42.62	+4.1	0.0214						
	9	43.4									
				A* = 0.46						A = 0.32	
3	Basal.	37.6				4	Basal.	36.2			
	1	39.25	38.56	+1.7	0.0431		1	38.18	36.92	+3.4	0.0531
	2	40.64	39.55	+2.5	0.0272		2	37.51	37.66	-0.4	0.0177
	3	39.86	40.58	-1.8	0.0194		3	37.31	38.41	-2.7	0.0099
	4	43.20	41.62	+3.8	0.0334		4	41.43	39.18	+5.7	0.0337
	5	41.11	42.69	-3.7	0.0176		5	39.38	39.97	-1.6	0.0168
	6	41.59	43.79	-5.2	0.0168		6	41.59	40.76	+2.3	0.0231
	7	40.85	44.91	+0.9	0.0117		7	43.07	41.58	+3.5	0.0240
	8	44.80	46.07	-3.0	0.0219		8	42.5			
	9	47.35									
				A = 0.60						A = 1.4	

5	Basal.	36.6				6	Basal.	31.7			
	1	37.44	+2.0	0.0426			1	33.70	32.75	+2.9	0.0613
	2	38.29	+0.4	0.0248			2	34.52	33.83	+2.0	0.0426
	3	38.78	-3.6	0.0193			3	37.04	34.86	+6.2	0.0519
	4	40.05	+0.2	0.0238			4	36.79	36.10	+1.9	0.0372
	5	40.77	-0.6	0.0216			5	37.64	37.29	+0.9	0.0343
	6	40.96	+1.0	0.0244			6	36.74	38.52	-4.7	0.0261
	7	41.90					7	38.88	39.79	-2.3	0.0282
							8	38.85	41.10	-5.5	0.0254
							9	42.38	42.47	-0.2	0.0321
							10	43.9			
			A=0.26							A=0.12	

* A = average aberration from the law.

Now $Q_t = Q_0 e^{kt}$ or by taking logarithms on both sides $\log_{10} Q_t = \log_{10} Q_0 + kt \cdot \log_{10} e$

In order to obtain the calculated heat value on a given day (*e.g.* 5th day) let the value for t correspond to the day. Therefore $t = 5$

$$\therefore \log_{10} Q_t = \log_{10} 37.4 + (0.0186 \times 5 \times 0.4343) \\ = 1.6133$$

$\therefore Q_t = 41.04 = \text{cal. calculated heat production per hr. per sq. m. on 5th day}$

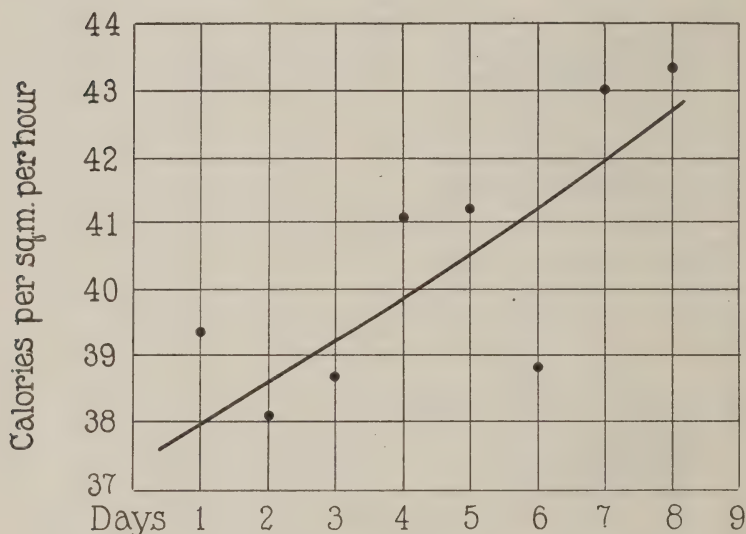


CHART 1. Case 1.

In each case the actual and calculated values were compared. The value for k in the data recorded here was obtained with t representing the last day of the experiment. It represents, not the number of days following the injection of the thyroxin, but the number of days from the 1st day (inclusive) any significant effect of the thyroxin was noted. For example in Cases 3, 4, and 5 a latent period of 1 to 2 days was noted.

In Table II are recorded in each case (*a*) the actual, (*b*) the calculated heat values, and (*c*) the percentage variation of the actual from the calculated. These are graphically represented in Charts 1 to 6. The continuous lines represent the calculated and the scattered points the actual values.

DISCUSSION.

It will be noted that the actual and calculated values, in the majority of instances, and the average aberration from the law agree within the limits of experimental error. However, in testing the validity of the assumption that the rate of heat production

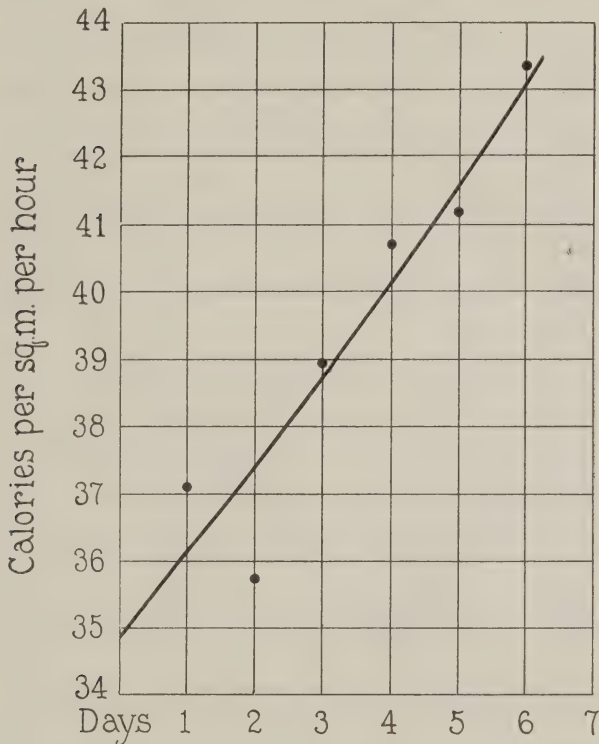


CHART 2. Case 2.

follows the law of multiple progression, theoretically, at least, the value for k in the equation

$$Q_t = Q_0 e^{kt}$$

obtained between intervals should, within the limits of experimental error, remain constant. It will be noted that in obtaining the "calculated" heat production at the different intervals, one value

for k was assumed to be constant and obtained as recorded above, and employed throughout the individual experiment. Thus:

Experiment 1	$k = 0.0163$
" 2	" = 0.0355
" 3	" = 0.0254
" 4	" = 0.0199
" 5	" = 0.0226
" 6	" = 0.0325

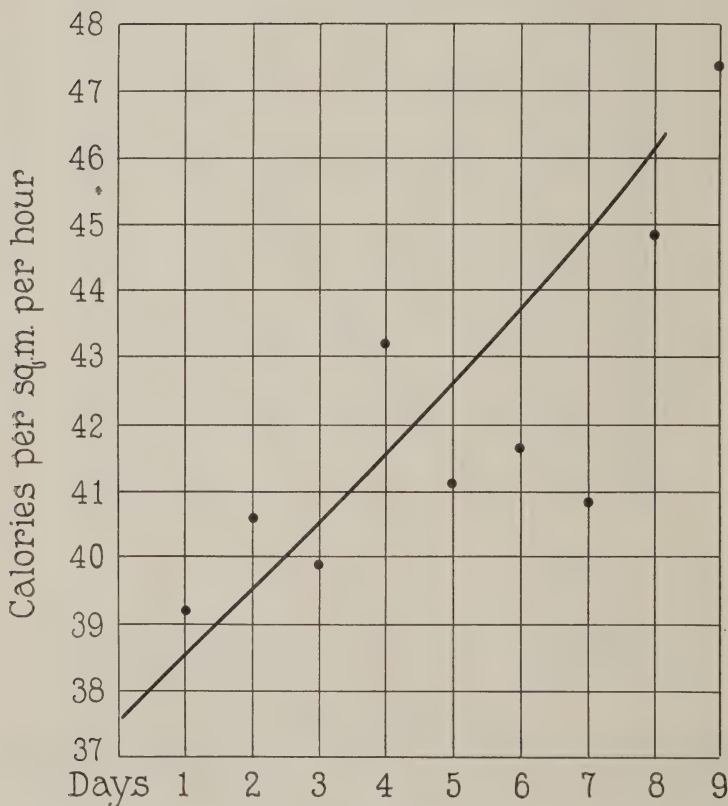


CHART 3. Case 3.

If the values for k are, however, calculated for different intervals a marked inconstancy is noted. The daily values in each case are recorded in Table II. From these data it is obvious that, as in many other physiological reactions, because the actual and

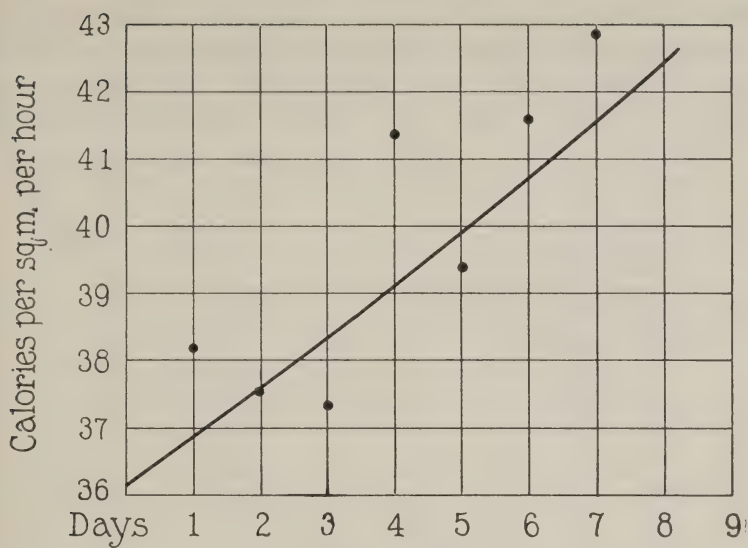


CHART 4. Case 4.

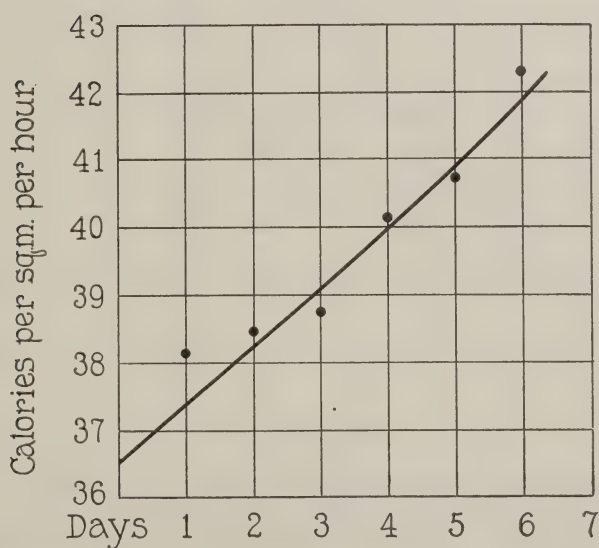


CHART 5. Case 5.

calculated values agree it does not necessarily follow that the explanation of the *complete* reaction may be found in this phenomenon. It will be noted that if the calculated (continuous) curve in each case were a straight line or convex to the left when plotted

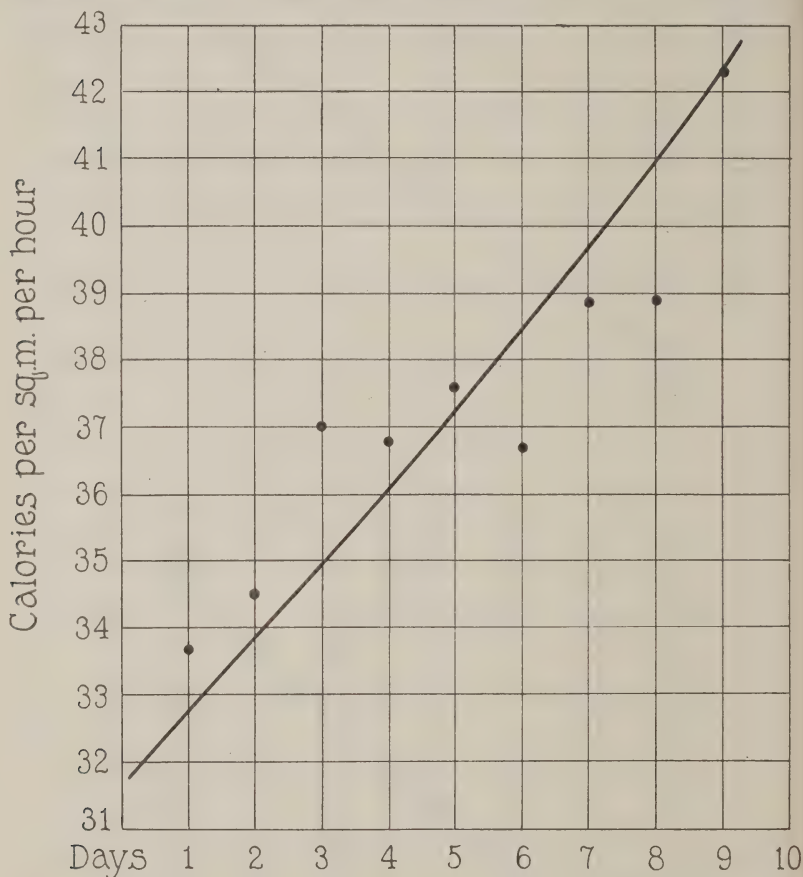


CHART 6. Case 6.

upon simple coordinate paper the actual and calculated values would still agree within the limits of experimental error. Therefore, the possible variations of the latter are apparently outside the limits within which one can specifically formulate the *complete* reaction. It appears hardly necessary to remark that by the term experimental error, as applied here, reference is made, not so much

to the technical procedure, as to the variations in metabolism due to other and more important factors, day to day variations, emotion, etc., in spite of the usual precautions to minimize them. All subjects were hospital patients.

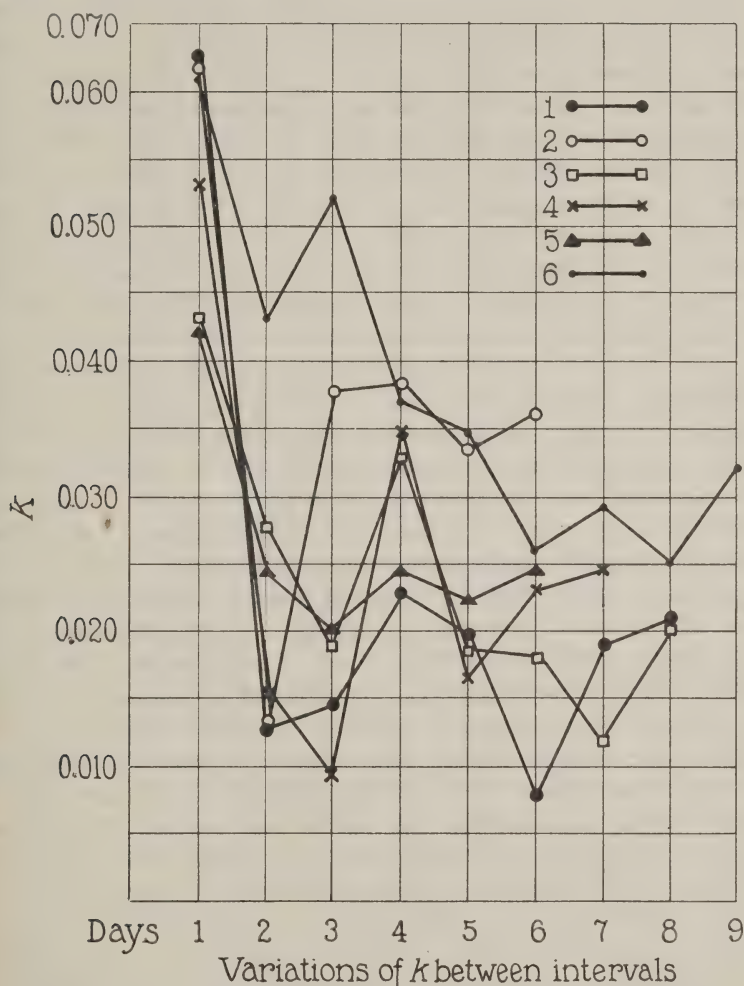


CHART 7.

The daily values of k in each case are plotted in Chart 7. A diminution in the value of k is noted during the early part of each experiment. It is not very likely that this phenomenon may

possibly be attributed to experimental difficulties involved in catching and determining conditions in the early part of the reaction, common to all chemical reactions, since the time factor here is not minutes but days. Since the values of k are not constant throughout the individual experiments, we are either not dealing with a reaction which follows the unimolecular law or, as in the case of ferments, the fall in k may be attributed to destruction of the catalyst. If, however, the latter explanation is accepted, it is difficult to reconcile the increase in the rate of heat production which follows with the view that the heat production is a direct function of the concentration of thyroxin in the tissues. Therefore, the conditions towards the end of the experiment (period of decay) do not appear to be those found during the earlier period.

These curves suggest other possibilities. The only positive evidence favoring the view that thyroxin is a catalyst is that in its presence the metabolism of the body, as indicated by the heat production, increases without any supply of energy from without. Though the maximum heat production appears to be a function of the amount of thyroxin administered and can approximately be calculated for given doses of the latter, there is no evidence to support the view that the same rate of metabolism would eventually be found if smaller doses were administered. Nor is there sufficient evidence that the thyroxin remains unaltered throughout the period of increased metabolism. Since the value for k decreases and the concentration of active protoplasmic mass may, relative to the dose of thyroxin, be regarded as constant, it is not impossible that the thyroxin is either being destroyed or altered. Part of the curves suggests autocatalysis, in that the coefficient of velocity increases in value towards the end of the experiments. Thus another possibility is that during the reaction certain compounds are formed which are catalytic and may account for the prolonged effect noted following administration of thyroxin.

In 1923, Boothby and Rowntree,² in a study of the effects of drugs on basal metabolism, recorded graphically a calorigenesis curve following thyroxin administration. The detailed data are not given. Although recalculations from a curve must obviously be approximate, the results are of sufficient interest to be com-

² Boothby, W. M., and Rowntree, L. G., *J. Pharmacol. and Exp. Therap.*, 1923-24, xxii, 99.

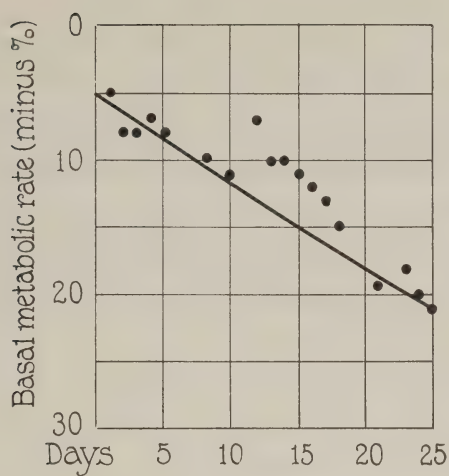


CHART 8.

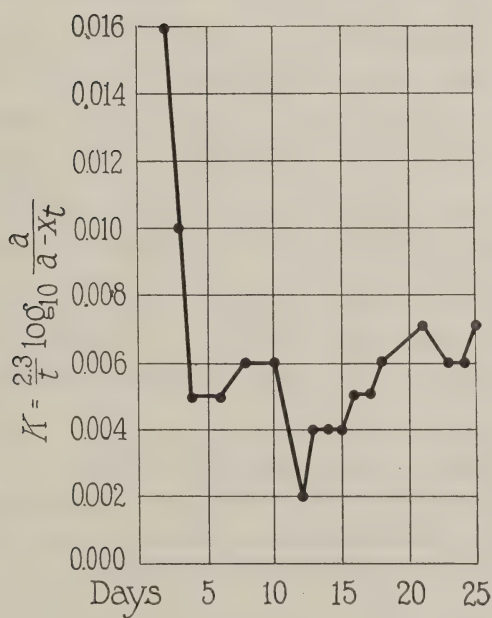


CHART 9.

mented upon. That the period of decay does not suggest a monomolecular reaction is obvious at a glance. The curve plotted on simple coordinate paper can only be regarded either as a straight line or concave to the left. When plotted on semilogarithmic paper, a straight line is approached. It need hardly be remarked that a straight line on semilogarithmic paper does not necessarily denote a monomolecular reaction.

Assuming the interpretation by Boothby and Sandiford of their curves to be correct, the loss of thyroxin takes place in accordance with the law for a monomolecular reaction. Therefore, as stated above

$$\frac{dx}{dt} = k (a - x) \text{ or}$$

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

The writer has calculated the various coefficients of velocity from this curve. The first value for k was obtained by t denoting the total number of days represented in the period of decay. The value for k was found to be 0.0073. When this constant is employed throughout the experiment, there is again noted an agreement between actual and calculated values within the limits of experimental error. The actual (scattered points) and calculated (continuous line) values are graphically recorded in Chart 8. However, when the values for k are obtained between intervals (daily), marked variations are noted. These are graphically recorded in Chart 9. Thus the data of the one curve of decay recorded by Boothby and Rowntree are essentially in agreement with those which the writer found in a study of the early part of the curve.

It is thus obvious from the above data that the metabolism immediately following the administration of thyroxin is complicated to such an extent by simultaneous side or secondary reactions that a specific coefficient of velocity for the reaction by the experimental method in use can hardly be calculated.

I wish to express my indebtedness to Miss Kathleen Wylde for technical assistance in this work.

THE RELATION OF NATURAL FOODSTUFFS AND THEIR TREATMENT ON GROWTH AND REPRODUCTION.*

BY H. G. MILLER AND W. W. YATES.

(From the Department of Agricultural Chemistry, Oregon Experiment Station, Corvallis.)

(Received for publication, August 11, 1924.)

In previous work reported by one of us (1), a ration satisfactory for the growth of young rats was used in which the potassium content was quantitatively controlled. This ration composed of purified foodstuffs plus water extract of yeast from which the potassium had been removed was satisfactory for growth when potassium salts were added. In our desire to obtain a ration satisfactory for growth and reproduction it was found necessary to substitute certain natural foodstuffs for the purified carbohydrates and proteins. The success obtained in reproduction when certain cereals were introduced into the diet as observed by investigators when they were studying the dietary qualities of these natural foodstuffs suggested the possibility of building a ration around one of these cereals, as corn, to accomplish our purpose.

By extracting corn with water it was thought it might be possible to reduce the potassium content below the minimum amount required for normal growth and reproduction and then by supplementing this corn with casein, salts, and vitamins to procure a ration capable of supporting growth and reproduction. While we were able to reduce the potassium to about 0.02 per cent by extracting with slightly acidified water which was below the amount previously found necessary for normal growth, this extracted corn did not substitute for whole corn in the diet in obtaining normal reproduction. In addition the results reported in this paper show the effect of supplementing certain basal rations with yeast, wheat embryo, alcoholic extract of wheat embryo, and kale.

* Published with the permission of the Director of the Oregon Experiment Station.

260 Foodstuffs on Growth and Reproduction

While this work was in progress our attention was called to the work of Evans and Bishop (2) in which they report an unknown fertility-conferring factor in wheat germ and certain other natural foodstuffs and which cannot be identified with vitamins A, B, C, and D. Later Sure (3) concludes from his work that there is a specific dietary factor which influences reproduction and suggests that it be termed vitamin E, although he attributes the success obtained by feeding wheat embryo as due to an increase in water-soluble B. From our own observations we are strongly inclined to agree with Evans and Bishop that wheat embryo contains some other dietary factor than water-soluble B which favorably influences reproduction.

TABLE I.

	Ash.	Crude protein.	Ether extract.	Crude fiber.	Starch.	Un- deter- mined.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole corn.....	1.52	10.22	2.56	2.26	70.23	13.22
Extracted corn.....	0.73	10.05	2.27	2.87	76.81	7.25

EXPERIMENTAL.

Animals from the stock group varying in age from 27 to 36 days were taken at the start of each experiment. For observing the effect of any supplementary factor, young from the same litter were used for the comparison. The rations used are given on each chart illustrating the growth curves and birth of young. The extracted corn was prepared by placing ground corn in muslin sacks and suspending them in water which had been slightly acidulated with hydrochloric acid (5 cc. of concentrated HCl to 10 liters of water). This was to cause coagulation of the proteins and as indicated in Table I the nitrogen content of the extracted corn was no less than that of the unextracted corn. The water was changed daily during a 4 day extraction period, after which the corn was removed and dried over steam coils. The deficiency of certain inorganic elements in cereals and the loss caused by water extraction were compensated by adding certain salt mixtures to the rations.

Salt No. 32 was used in former work (1). In this work two other salt mixtures whose compositions are given below were made up so

that the sodium and potassium could be altered without changing the concentrations of the other elements. We are aware that it was not necessary to add all of the inorganic components in these salt mixtures to certain of the rations, but it was done to tend towards uniformity in respect to the inorganic portion of the diets.

The rations were fed *ad libitum* and no record was kept of the individual intake of each animal. We think the performance of each group of animals as a whole was sufficient to bring about any difference in the dietary qualities of the particular ration employed.

Certain groups were allowed shavings for litter while most of the animals ran on screens. An effort was always made to isolate the pregnant females and provide them with filter paper or shavings for a nest.

Composition of Salt Mixtures.

<i>Salt B (K-free).</i>		<i>Salt C.</i>	
	<i>parts</i>		<i>parts</i>
NaCl.....	60	KCl.....	71
MgSO ₄	60	MgSO ₄	60
Na ₂ HPO ₄	100	Na ₂ HPO ₄	100
CaHPO ₄ ·2H ₂ O.....	250	CaHPO ₄ ·2H ₂ O.....	250
Ca lactate.....	60	Ca lactate.....	60
Fe citrate.....	25	Fe citrate.....	25

DISCUSSION.

The marked effect on reproduction by extracting corn with water is apparent in Charts 1 to 15. Whether the water actually removes some dietary factor necessary for reproduction or if in the process of extracting and drying there is an alteration in certain compounds directly connected with the reproductive capacity remains to be investigated. Experiments by Mattill and Conklin (4) and Mattill and Stone (5) who failed to secure normal growth with rats with milk as a sole source of diet and Palmer and Kennedy (6) who obtained normal growth and reproduction on artificial dried milk show the effect of changing the properties of a natural foodstuff providing the dietary factors did not vary in quality and quantity in the original milk used by these investigators. This difference in dietary properties most likely explains the difference in results obtained by Nelson, Heller, and Fulmer (7) and Evans and Bishop. The yeast used

by the former investigators probably contained another dietary constituent not present in the yeast used by Evans and Bishop or ourselves. Differences in results from feeding natural foodstuffs are also exemplified in the work of Sure (8) who reports lack of fertility and significant success in rearing of the young on milk diets while Anderegg (9) reports that growth and reproduction result when whole milk powder is the only source of protein and vitamins in the diet.

From our observations on feeding wheat embryo and yeast at different levels to young rats, the former was not richer in water-soluble B than the yeast as shown in the growth curves (not illustrated here). However, when they were fed at levels of 5 and 10 per cent, wheat embryo or the alcoholic extract of the same always favored reproduction. The growth curves of the animals in Group 39, Chart 14, on 4 per cent yeast correspond to those of Groups 38 and 40. The reproduction in Group 39 must have been due to the small amount of kale received during the first 4 weeks. Groups 38 and 40 receiving 4 and 10 per cent of yeast, respectively, showed no signs of reproduction. 10 per cent wheat embryo substituted for the 10 per cent yeast resulted in reproduction as shown in Group 31. The failure of the wheat embryo-fed rats to rear their young is difficult to explain. Certain litters appeared to grow normally up to the weaning time and then lost weight and died. Others after a period during which the weight remained constant would grow normally. Toxic substances in wheat embryo reported by McCollum and Davis (10) may be the cause of this disturbance. However, one group given milk with the wheat embryo grew normally.

The failure of a female rat to rear one litter is no indication of a similar failure in the next litter. We have observed that some animals on a ration which we consider adequate in all the dietary requirements would lose their young while they would successfully rear the next litter. This was more noticeable with the animals on screens than those on shavings.

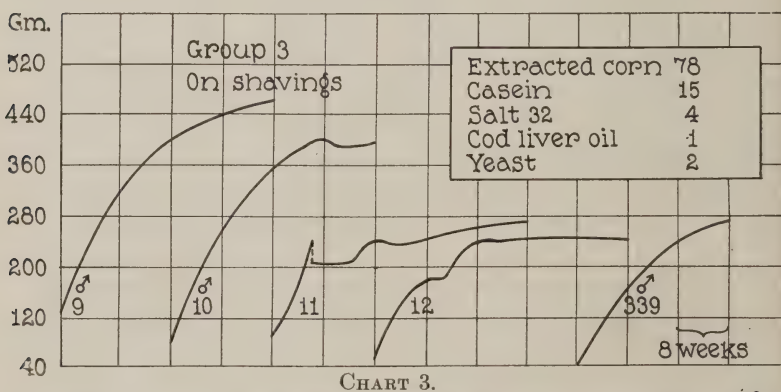
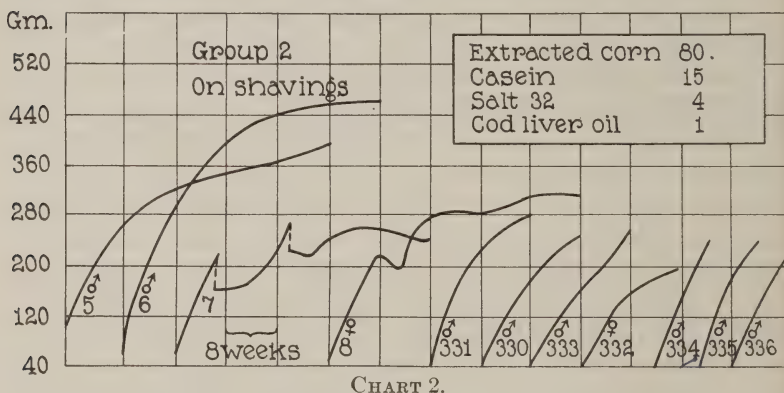
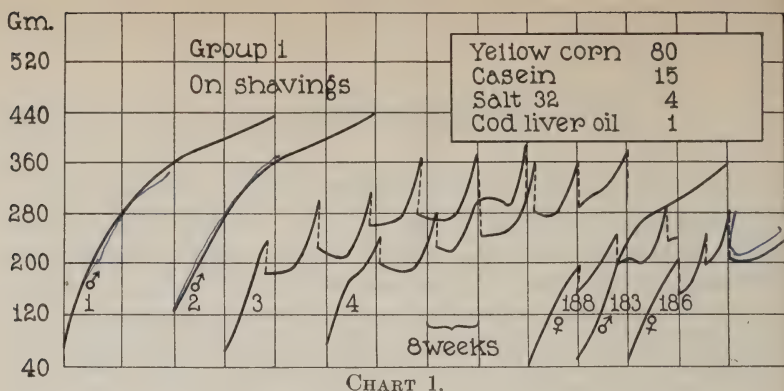
CONCLUSIONS.

Corn extracted with cold water and then dried (not over 100°C.) did not contain the dietary factors necessary for reproduction.

Unextracted corn, wheat embryo, alcoholic extract of wheat embryo, and green kale contain this dietary factor.

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CHARTS 1, 2, and 3. The marked difference in reproduction resulting in substituting extracted corn for the whole corn is shown. No. 7 had two litters of 10 and 5 young, 9 out of the 11 left with the mother were weaned and averaged 45 gm. in weight at the end of 35 days. From then on the growth curves of Nos. 330 to 332 from the first litter and Nos. 333, 334, and 336 representing the second litter appear normal, but the one female showed no signs of pregnancy. Even the males did not mate with females from the breeding colony. No. 11 in Group 3 had one litter of 9 but raised only one (No. 339) of the 6 allowed to remain with the mother. No. 339 did not mate with fertile females. During the same time Nos. 3 and 4 in Group 1 gave birth to 45 and 47 young, respectively, and weaned all that were not removed. At the end of 30 days the young averaged from 50 to 70 gm. in weight. Nos. 183, 186, and 188 illustrate the performance of animals in the third generation, their young averaged 45 to 70 gm. in weight at the end of 32 days.

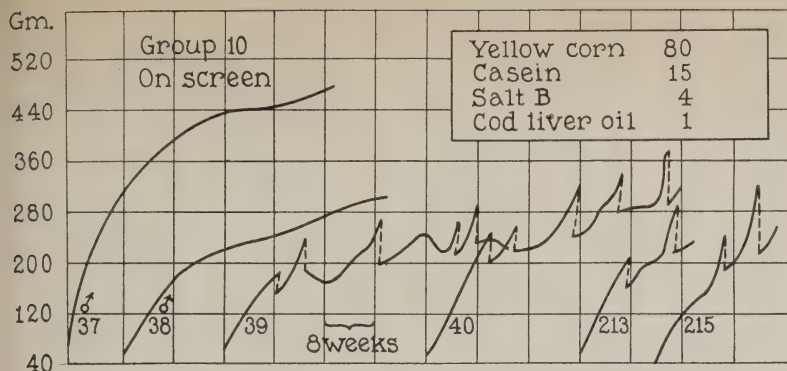


CHART 4.

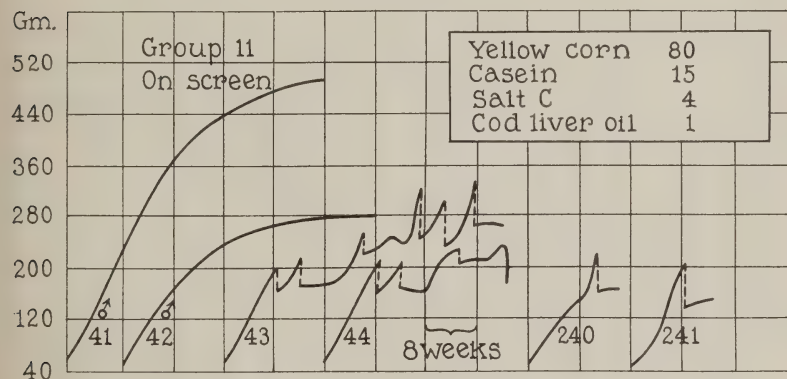


CHART 5.

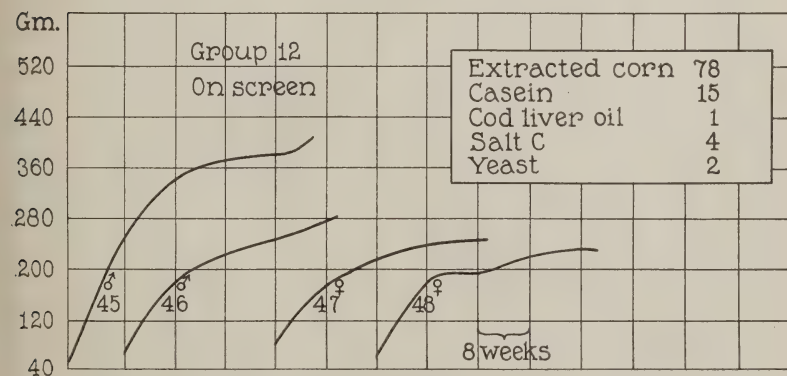


CHART 6.

CHARTS 4, 5, and 6. No reproduction in Group 12 receiving extracted corn compared to Groups 10 and 11 again demonstrates the effect of this change in diet. As indicated Salt Mixtures B and C were used and the animals placed on screens. In Group 10, 79 young were born while in Group 11, 68 young were born. About 60 per cent of the young not removed from the mother were reared. No. 44 died for some unknown reason before the experiment was terminated. The young weighed on the average from 45 to 65 gm. at the end of 32 days. Nos. 213, 215, 240, and 241 are examples of growth and reproductive ability in the third generation. Their young were also successfully reared.

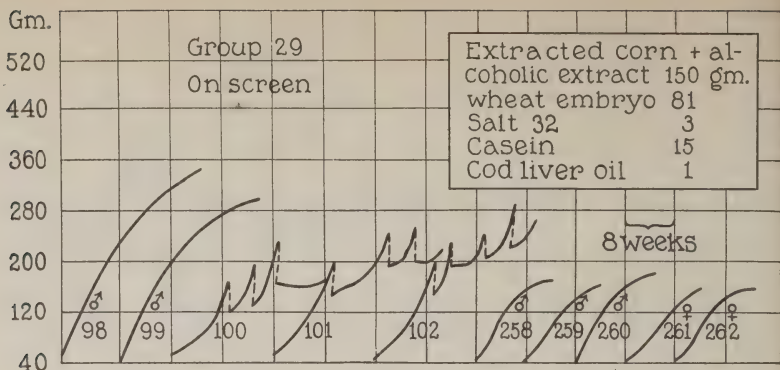


CHART 7.

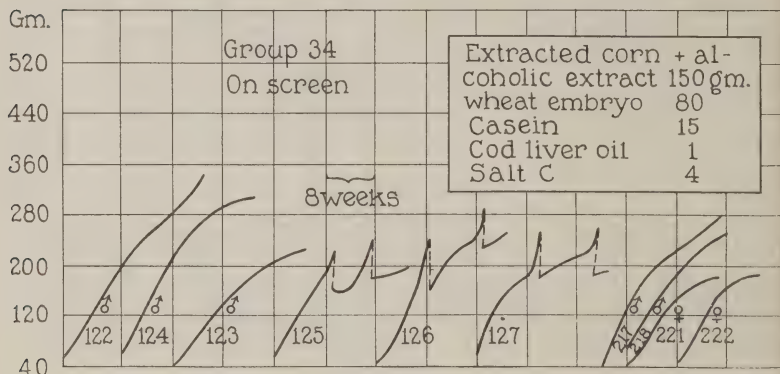


CHART 8.

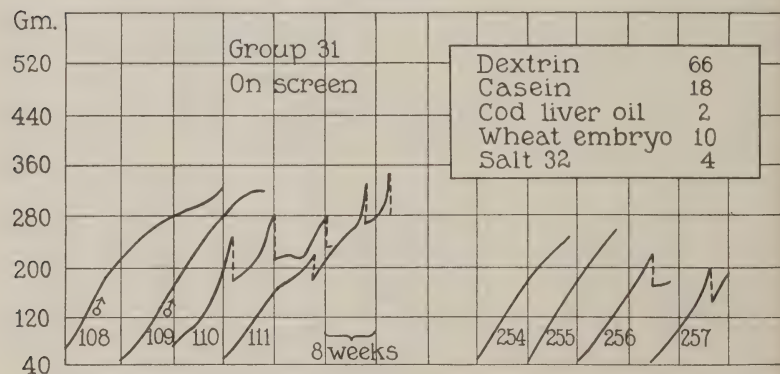


CHART 9.

CHARTS 7, 8, and 9. Where the rations were supplemented with wheat embryo or an alcoholic extract of wheat embryo normal periodic reproduction occurred, but rearing of the young was unsatisfactory. Out of 70 young born in Group 29 only 5 were raised and these were from the same litter. They averaged 53 gm. in weight at 46 days of age and as indicated in Chart 7, Nos. 258 to 262, the growth curve was below normal and there was no reproduction. Of the 43 young born in Group 34, 8 were raised, averaging 45 gm. at 40 days. They then grew normally, but did not reproduce as shown by Nos. 217, 218, 221, and 222. The two females in Group 31 gave birth to 39 young, but only 9 were raised. However, there was reproduction in the second generation, Nos. 254, 255, 256, and 257, but only 1 was raised out of 14.

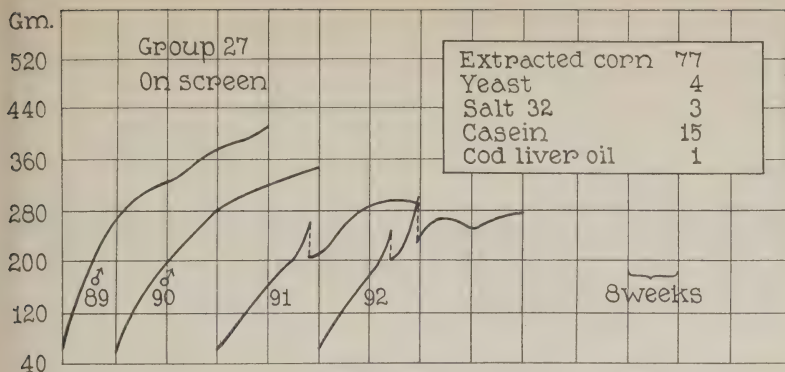


CHART 10.

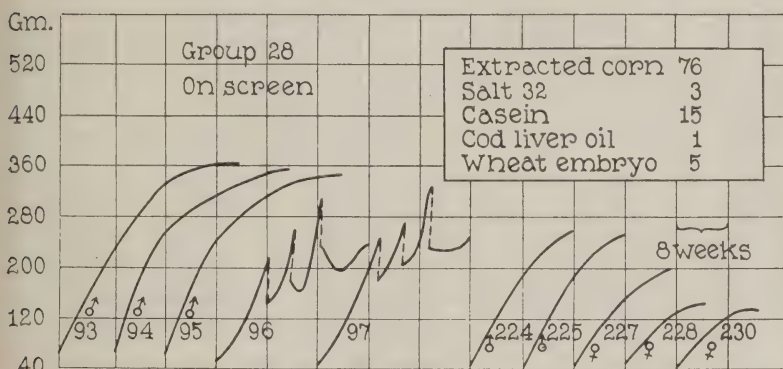


CHART 11.

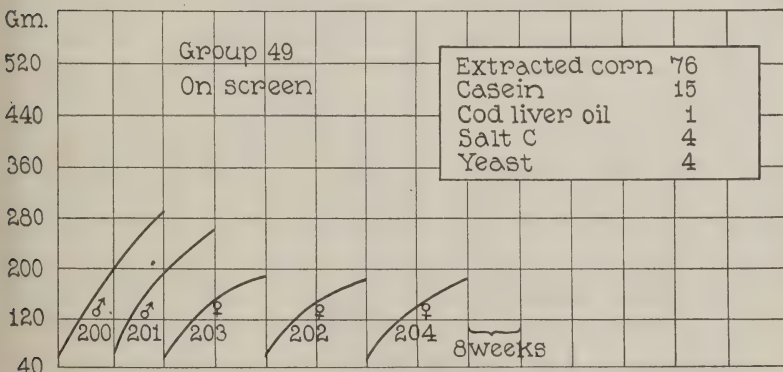


CHART 12.

CHARTS 10, 11, and 12. These charts further show how wheat embryo influences reproduction. The same result was not obtained by adding yeast. None of the young born on the yeast ration was raised and reproduction did not continue as in Group 28; the second generation of this group did not reproduce.

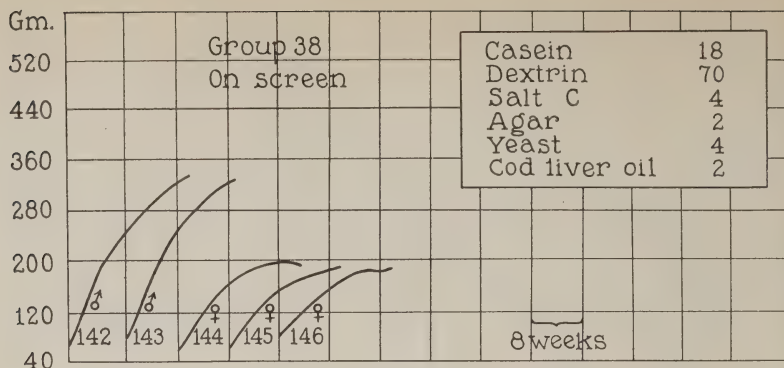


CHART 13.

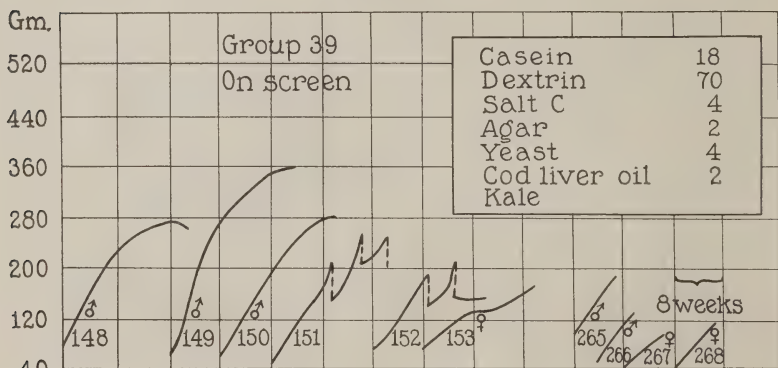


CHART 14.

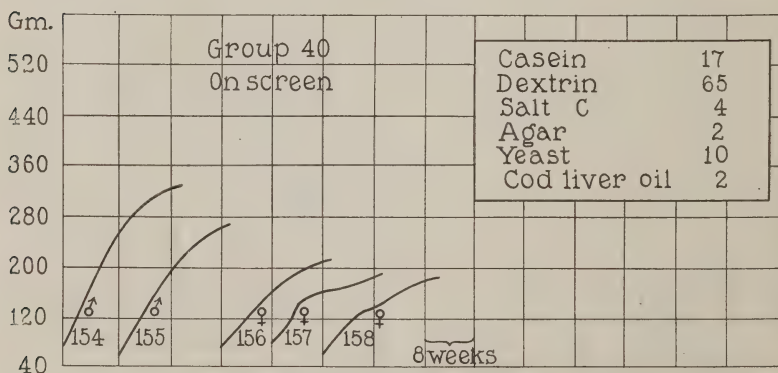


CHART 15.

CHARTS 13, 14, and 15. Group 38 shows normal growth on this ration but no reproduction. Increasing the yeast to 10 per cent caused no improvement in appearance or reproduction in Group 40. The reproduction and raising of one litter in Group 39 were undoubtedly due to the 20 gm. of green kale fed daily during the first 4 weeks of the experiment. It was discontinued because the kale was unobtainable. The growth of the second generation was below normal. The five animals weighed 120 gm. at the end of 32 days, 325 gm. at the end of 60 days; from then on their growth curve is indicated on the chart.

THE CARBON DIOXIDE EQUILIBRIUM IN ALVEOLAR AIR AND ARTERIAL BLOOD.*

BY A. V. BOCK AND H. FIELD, JR.

(From the Medical Laboratories of the Massachusetts General Hospital,
Boston.)

(Received for publication, September 5, 1924.)

In a recent paper (1) we expressed the opinion that the CO_2 tension of alveolar air was best represented by that of direct samples collected by the Haldane-Priestly method, but at the end of normal expiration only, rather than by the average of the CO_2 tensions of samples collected at the end of both expiration and inspiration. We also stated our belief that there is, in ordinary circumstances, nearly complete CO_2 equilibrium between alveolar air and arterial blood. The purpose of this paper is to discuss the matter in more detail and to present further data.

There have been various methods used for determining the gases of the alveolar air. Discussions have been published concerning their accuracy and the relation of the values obtained to the actual alveolar air at different times in the respiratory cycle and to the average alveolar air. These discussions dwindle in importance as one realizes that the alveolar air is a variable thing, changing from minute to minute with variations in the pulmonary ventilation, that the respirations are apt to be distinctly abnormal while a subject is connected with an apparatus so that alveolar air values obtained in this manner have an indeterminable relation to the alveolar air of the subject while he is breathing normally, and that the alveolar air has but little significance except in connection with other functions. The generally useful figure is the effective alveolar air—the gas tensions that control diffusion between the blood and the lung air—not the alveolar air at any particular time in the respiratory cycle or indeed the average alveolar air.

* The expenses of this research were defrayed in part by the Proctor Fund and by the Tutorial Fund of Harvard University.

We believe that we have obtained some information on this subject by direct experimentation, having had occasion, in the course of other studies (1, 2) to make determinations of alveolar and arterial CO₂ tensions as nearly simultaneously as possible. Inasmuch as determinations could not be made exactly simultaneously, the relationship of the values obtained in the individual experiments varies somewhat. It would seem, however, that the data presented are extensive enough so that statistical treatment of them warrants fairly definite conclusions.

Our experimental methods have been described elsewhere (1, 3) and need not be discussed in detail. Arterial puncture was performed under novocaine anesthesia. Generally, anesthesia was complete enough so that the subject was not aware when blood was obtained. The probability of the effect of abnormal breathing upon the blood gases was thus reduced to a minimum. Points for the construction of the CO₂ dissociation curve were obtained by the analysis of both blood and gas equilibrated in tonometers in a water bath maintained at 37.5°C. In the majority of instances arterial blood was used for this purpose. When sufficient arterial blood was not obtained, venous blood, drawn without stasis, was used. In the instances in which both arterial and venous bloods were used, no significant difference between the two was detected. The alveolar CO₂ tensions quoted are the averages of those of several samples, usually from 4 to 6, collected as close, in point of time, to the arterial puncture as possible. They were obtained with the usual rubber tube equipped with a sliding valve. Inasmuch as it became apparent early in the investigation that the CO₂ tensions of samples collected at the end of normal expiration agreed fairly closely with those of arterial blood and that the CO₂ tensions of samples collected at the end of normal inspiration were invariably a few millimeters of mercury lower, the collection of the later samples was soon abandoned and only the expiratory samples are quoted.

The data of these experiments are presented in Table I. In all experiments except three, those of J. M. F., of S. L. W., and the fourth observation on Case 3, there was less than 2 mm. of mercury difference between the CO₂ tensions of alveolar air and arterial blood. In fifteen of the experiments the difference was 1 mm. of mercury or less. The average of all except the three experiments

mentioned (and in these the direction of variation was divided) shows an alveolar CO_2 tension 0.48 mm. of mercury below that of the arterial blood.

That there actually is a difference between the alveolar and arterial CO_2 tensions is probable, not so much because of slowness of CO_2 diffusion or the need of a pressure head, but, as L. J. Hen-

TABLE I.

Subject.	Experiment No.	Alveolar CO_2 .	Arterial CO_2 .	Difference.
		<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>
F. W. L.	1	40.7	41.1	+0.4
	2	41.0	41.75	+0.75
F. T. H.	1	38.0	38.0	0
C. M. J.	1	38.6	39.6	+1.0
	2	39.9	41.4	+1.5
J. M. F.	1	35.45	40.1	+4.55
A. V. B.	1	37.1	36.2	-0.9
	2	37.1	37.7	+0.6
H. F.	1	37.13	37.5	+0.37
	2	37.2	38.6	+1.4
	3	37.4	38.7	+1.3
	4	35.6	35.2	-0.4
	5	37.2	37.5	+0.3
S. L. W.	1	39.5	36.0	-3.5
H. P. S.	1	42.9	43.15	+0.25
Case 2.	1	26.4	26.2	-0.2
	2	34.2	35.7	+1.5
Case 3.	1	26.0	27.2	+1.2
	2	37.8	37.0	-0.8
	3	39.8	40.7	+0.9
	4	38.0	40.7	+2.7
Case 4.	1	23.5	23.8	+0.3
	2	16.9	17.2	+0.3
	3	19.7	20.1	+0.4

derson pointed out,¹ because of slowness of O_2 diffusion. The latter is slow enough so that oxygenation is continuing during the passage of blood through the pulmonary capillaries and is not complete when it leaves them. Oxygenation of blood increases its CO_2 tension and the final adjustment for the last increase in CO_2 tension due to oxygenation is probably not complete as the

¹ Personal communication.

blood leaves the capillaries. This tension difference, however, must be small, very likely somewhat less than the 0.48 mm. of mercury observed, and it is extremely unlikely that it is as much as 1 to 2 mm. of mercury, which would have been the finding if inspiratory and expiratory alveolar air samples had been averaged.

Aside from the experimental evidence presented, there are good theoretical grounds for the belief that there can never, except in case of pulmonary pathology or cardiac defect, be any considerable difference between the CO₂ tensions of alveolar air and arterial blood and that the effective alveolar air is approximately that of the Haldane-Priestly sample collected at the end of normal expiration.

Van Slyke (4) pointed out that, inasmuch as CO₂ diffusion is in the neighborhood of twenty to thirty times as rapid as O₂ diffusion, failure of CO₂ equilibrium between alveolar air and arterial blood can hardly occur while conditions are such as to permit proper oxygenation of the latter.

We believe that the discrepancies between the CO₂ tensions of alveolar air and arterial blood of normal subjects reported by previous workers are due to variations in the respirations immediately preceding the collection of the alveolar air samples or while the arterial blood is being obtained. That the discrepancies are real is hardly possible for the reason stated above. That they are due to inadequate alveolar air samples is improbable as Haldane (5) has shown that with expirations greater than 800 cc. the depth of the expiration has practically no effect on the CO₂ content of the sample obtained.

The reason that the CO₂ tension of samples of alveolar air obtained at the end of inspiration is so much lower than that of the effective alveolar air becomes apparent upon consideration of the movements of air in the respiratory passages.

Douglas and Haldane (6), as other authors have done, observed, in experiments where dilution of the alveolar air by artificial gas mixtures was attempted, that by merely taking a single breath of the gas an even mixture in the lung alveoli did not result. It is not probable that fresh air inhaled in a normal inspiration will mix more completely or more quickly with the alveolar air than did the artificial gas mixtures.

Y. Henderson, Chillingworth, and Whitney (7), in studies of the movements of gases in tubes, demonstrated that smoke blown into the end of a glass tube did not move along it in a cylindrical column, but in the form of a thin cone or "spike" which, when its tip had reached the end of the tube, filled not more than one-half or one-third of it. When the smoke stream was stopped the "spike" immediately broke and mixture with the surrounding air took place, the smoke and air mixture being more dense at the base of the cone than at the top.

If the movements of air in the respiratory tract occur in such a manner, when the lung expands and each alveolus enlarges, fresh air enters the respiratory passages in the form of an axial stream. As it proceeds, this stream gradually enlarges at its base so that the larger air passages are completely washed out by it. Considering the proportion of the size of the usual inspiration to the capacity of the lungs, only the tip of the advancing "spike" of fresh air enters the expanding alveolus and fills only a fraction of it. Correspondingly, on expiration, air is expelled first from the proximal and central portions of the alveoli.

At the end of inspiration, diffusion occurs between the central core of fresh air and the surrounding alveolar air. This diffusion can hardly be instantaneous and the air in an alveolus is probably never a uniform mixture during normal respiration. There is, in the center, a comparatively large proportion of fresh air while, as the alveolar wall is approached, the CO_2 content gradually increases and the O_2 content diminishes. The deepest expiration leaves a large volume of air in the alveoli and the residual air is the portion that was originally nearest the alveolar walls. Hence it is that the Haldane-Priestly sample of alveolar air taken at the end of inspiration contains slightly less CO_2 than the air in contact with the alveolar walls which controls the arterial CO_2 tension and is the effective alveolar air. During the time taken by expiration diffusion becomes more complete and the sample taken at its end more closely approaches the effective alveolar air.

Both Haldane (5) and Y. Henderson, Chillingworth, and Whitney (7) have produced evidence to show that indirect methods of determining the alveolar air are liable to error because of variations in the dead space with variations in the depth of the respirations. The above discussion makes clear another source of error. The

air expelled from the alveoli during expiration contains a larger proportion of fresh air than does the total or the effective alveolar air. Consequently, calculations based upon it, other factors being correct, must give values that are too low for CO₂ and too high for O₂.

SUMMARY.

In twenty-one experiments the average CO₂ tension of arterial blood was 0.48 mm. of mercury greater than that of Haldane-Priestly samples of alveolar air taken at the end of normal expiration.

Haldane-Priestly samples of alveolar air taken at the end of inspiration have a CO₂ tension that is a few mm. of mercury lower and the average of the CO₂ tensions of the inspiratory and the expiratory samples is somewhat less than that of the effective alveolar air.

This is explained by the peculiarities of the movements of air in tubes.

Another source of error in indirect methods of determination of alveolar air is pointed out.

CONCLUSIONS.

The CO₂ tension of alveolar air approaches very closely that of arterial blood in all except subjects having pulmonary pathology or cardiac defect.

The effective alveolar air is very closely represented by the Haldane-Priestly direct sample obtained at the end of expiration.

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FAT-SOLUBLE VITAMINS.

XX. A MODIFIED TECHNIQUE FOR THE DETERMINATION OF VITAMIN A.*

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PLATE 2.

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Some time ago we had occasion to point out the fact that millets are not uniformly rich in the so called fat-soluble vitamin because on a diet carrying millet supplemented with purified casein and salts we obtained growth on some varieties but not on others (1). At that time this was the only interpretation which could be made of our experimental findings because there were no other factors known to which, under the experimental conditions imposed, responsibility for the failure of growth could be attributed. It is now known, however, that the growth-promoting properties of the so called fat-soluble vitamin are in reality due to at least two substances, one functioning in preventing infection of the respiratory tract and eyes,—and now designated as vitamin A,—and the other as an antirachitic agent, now called the antirachitic vitamin (2). It is from the standpoint of the occurrence of this antirachitic vitamin, under certain conditions just as indispensable for growth as vitamin A, that our former conclusions must be reevaluated. Parenthetically, it may be stated that this has again shown that there is no occasion for speaking of vitamin A as “the growth vitamin” as has been customary in certain English laboratories.

To our mind the discovery that a lack of the antirachitic vitamin may be responsible for growth inhibitions under certain con-

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ditions has completely invalidated the sweeping conclusions that have been made with respect to the non-occurrence of "the fat-soluble vitamin" not only in millets but in other materials as well where growth has been used as the sole criterion.

A reinspection of our data obtained with the millets supported the idea that those millets which had not allowed normal growth had not done so because of their deficiency in the antirachitic vitamin. This appeared true for two reasons. In the first place, the presence of rickets was indicated because some of the rats from the aforementioned experiments had been observed to walk with a shambling gait, resembling rickets, which was not observed in the lots which grew; and in the second place, a sufficiently liberal supply of vitamin A to prevent interference with growth was suggested because in no case was an ophthalmia observed previous to the 10th or 12th week, when normally in our animals on the ration free from vitamin A, it usually makes its appearance by the 6th and rarely, if ever, after the 8th week. All in all these observations made it appear highly desirable that further experiments should be carried out. The result has been that we have seen fit to modify the growth method heretofore in use for the determination of vitamin A, and, incidentally, we have accumulated some data bearing on the quantitative occurrence of vitamin A not only in millets but in other materials as well.

EXPERIMENTAL.

In view of what has been said, in order that the full growth-promoting potentialities of vitamin A may be allowed to manifest themselves, it is necessary to satisfy in some way or other the requirements of the animal for the antirachitic factor. This is possible by the use of aerated cod liver oil or else by direct irradiation of the animals with ultra-violet rays. The latter appeared to us to be the preferred way because of the exclusion of the possibility of contaminating the animal's ration with vitamin A and also because cod liver oil in all forms, even when oxidized to destroy vitamin A, is not greatly relished by the animal. In view of recent results obtained from our studies of the action of light in relation to the antirachitic vitamin, the use of light as the equivalent of the vitamin appeared to be an entirely rational procedure.

For irradiation, as a source of light, we used a Cooper-Hewitt BY type quartz mercury vapor lamp, run at a voltage of 40, at 4.5 amperes. The rats were exposed at a distance of about 22 inches from the lamp for 10 minutes daily, 6 days of the week.

In using light for these experiments it was necessary to rule out the storage factor for both vitamin A and the antirachitic vitamin by determining first of all what results in the way of growth might be expected from our young experimental animals when put on a ration free from fat-soluble vitamins. Without such information available it would obviously be impossible to determine from the growth performance of the rats what degree of response could be attributed directly to the unknown vitamin content of the ration. For this reason young rats similar to those used for later experiments were tested out for their fat-soluble vitamin reserves, using identical methods.

For the basal ration we used a mixture of purified food constituents consisting of alcohol-extracted and heated casein 18, agar 2, yeast 6, salt 40 (2) 4, and dextrinized starch to make 100. When materials very potent in vitamin A and the antirachitic factor were tested they were added to the ration at the expense of so much dextrin. For materials not so potent, such as grains, supplements of only casein and salts were used,—the other constituents were omitted. In all cases it is to be noted that there was given a liberal allowance of a salt mixture rich in calcium and in phosphates, which latter is of great importance because it has a direct bearing upon the results obtained.

The rats used in the experiments were young, white and pied animals, ranging in weight from 40 to 59 gm., and from 20 to 25 days in age. They were raised with the usual care in our stock colony on our standard stock ration (3) and were selected for uniformity as to state of nutrition. On the experiments they were confined in our standard laboratory cages provided with false screen bottoms (4), four animals to a group in each case.

All animals were weighed weekly and notations as to general condition made daily or as often as impending change of condition made it desirable. With the termination of the experiment a humerus and femur were dissected out and dried at 96°C. and extracted for 24 hours with hot alcohol. After drying and weighing they were ashed in an electric muffle furnace to a white ash to obtain percentage of ash.

Chart I brings out by the virtual absence of growth in the non-irradiated animals and the time of incidence of ophthalmia in all of them that neither vitamin A nor the antirachitic vitamin were held in excessive storage by our young animals. Yet, at first sight, it might appear as though the amount of vitamin A could have been reduced with profit. Past experience, however, has shown us the inadvisability of feeding a stock ration inordinately

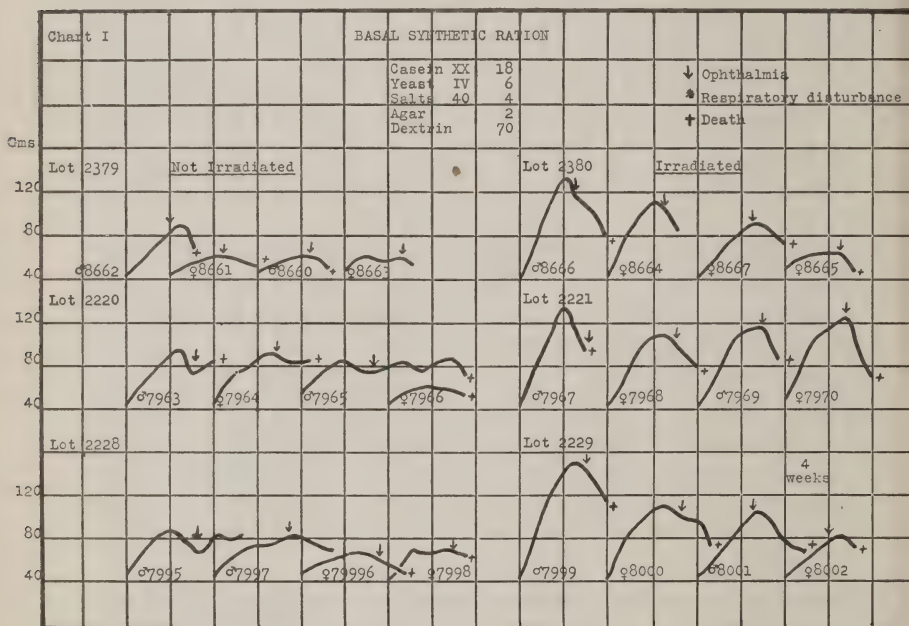


CHART I.

low in vitamin A because a deficiency of vitamin A in the ration of the unweaned young is rarely indicated by ophthalmia. As a result, the prevalence of vitamin A deficiency may escape detection for a considerable time, jeopardizing the continuous supply of suitable experimental animals. The fact that growth for the first 4 weeks was normal in many of the irradiated animals goes to show that the experiments for the determination of vitamin A must be run for at least 8 weeks and preferably 10.

Table I brings out the difference in ash content of the bones of irradiated and non-irradiated animals on the vitamin-free ration. As some of these animals were the same as those shown in Chart I it is seen that with the increased growth as a resultant of irradiation increased calcification of the bones occurred simultaneously. This supports the idea of an interrelation of growth to the anti-rachitic process; although it does not prove it, as they may be merely independent concomitant resultants of the irradiation.

In Chart II are shown the first data obtained on a ration of unknown vitamin content, testing out the hypothesis that failure of growth in our earlier hog millet experiments was due to lack of antirachitic vitamin rather than to lack of vitamin A. This hy-

TABLE I.
Percentage of Ash in Bones of Rats on Basal Synthetic Ration.

Lot No.	Rat No.	Sex.	On ration..	Not irradiated.		Irradiated.	
				Humerus.	Femur.	Humerus.	Femur.
			<i>wks.</i>				
2224	7982	♀	10	49.22	47.02		
2225	7983	♂	10			55.41	53.80
2228	7995	♂	10	47.77	49.20		
2229	8001	♂	10			52.38	
2379	8661	♀	9	46.9	42.7		
2380	8666	♂	8.5			50.6	50.0
2379	8663	♀	6.5	47.6	48.5		
2380	8664	♂	6.5			50.8	50.3

pothesis is supported by the data here presented although unfortunately two of the non-irradiated animals died very early—No. 7729 showing a rachitic walk and No. 7730 even being seized with occasional tetany suggesting rickets though their autopsy and x-ray examinations were overlooked. It is certain that they did not die from lack of vitamin A as respiratory symptoms and ophthalmia were absent. The two remaining animals at the end of 3 weeks averaged 73 gm., the four irradiated, on the other hand, averaged 203 gm.

Plate 2 shows radiographs taken of Rats 7732 (non-irradiated) and 7742 (irradiated) after they had been on the ration for 17 weeks. It brings out the diminutive size as well as the deficient calcification and rachitic metaphysis of the non-irradiated animals

in contrast with the irradiated. Frequently, however, rachitic metaphyses are not observed in the non-irradiated animals even though growth may be inhibited by the absence of the antirachitic factor. We have already frequently pointed out how rickets does not always manifest itself histologically—the latter resulting only through a peculiar combination of factors including growth, absence of the antirachitic agent, and a deranged calcium and phosphorus assimilation. The specific rôle of each of these in the production of the rachitic picture is not yet understood, but it is evident that there may be a deficiency of antirachitic vitamin with absence of production of the characteristic histologic or radiographic picture.

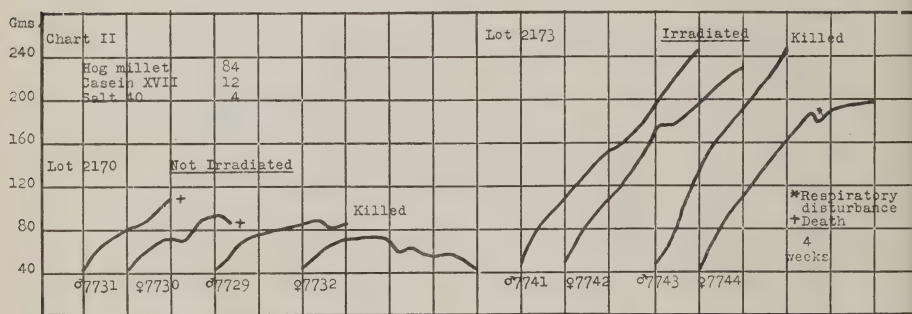


CHART II.

Table II shows the wide discrepancy in the mineral content of the non-irradiated as compared with the irradiated animals. With the failure of growth and the formation of rachitic metaphyses calcification was only two-thirds as complete as in the irradiated.

The criticism that applies to our early experiments on millet has indeed a very general application where growth has been used as the criterion of the amount of vitamin A present in a food-stuff or feed. Chart III presents some data obtained with alfalfa. In these experiments alfalfa was incorporated at 4.0, 2.0, 1.5, 1.0, 0.8, 0.5, and 0.0 per cent levels in our usual basal synthetic ration of purified foodstuffs and yeast. The alfalfa used represented the third cutting of the season. It was cured in the dark in a well ventilated room and then ground to a meal. Each group of animals was run in duplicate, the duplicate series being irradiated.

To eliminate variations due to difference in animals the litters were divided among the various groups. Chart III presents the data only in part.

The control group without alfalfa presents results approximately the same as those shown in Chart I on the same ration—the outstanding feature of the whole experiment being the normality of the growth obtained even on the low levels of intake of alfalfa with irradiation. Though not shown on the chart, even 0.5 per cent of alfalfa allowed normal growth to take place when the animals were irradiated. Without irradiation approximately 4 per cent of alfalfa was necessary to effect normal growth. We attribute these results to the fact that but little alfalfa is necessary to furnish the animal enough vitamin A for normal growth, but that—light excluded as the antirachitic agent—at least 4 per cent

TABLE II.
Percentage of Ash in Bones of Rats on Hog Millet.

Lot No.	Rat No.	Sex.	On ration. <i>wks.</i>	Not irradiated.		Irradiated.	
				Humerus.	Femur.	Humerus.	Femur.
2170	7729	♂	12	40.70	41.68		
2173	7743	♂	12			60.54	62.72
2170	7732	♀	17	35.61	40.34		
2173	7742	♀	17			62.94	61.58

is necessary to furnish enough of the antirachitic factor. This point of view is supported by the data of Table III because they show that at the lower levels of alfalfa intake calcification of the bones was not normal.

There appears to be no question that recognition of the disturbing effect of lack of the antirachitic factor is necessary in vitamin A studies, but in addition our experiments have opened up the question of simultaneous determination of the distribution of the antirachitic vitamin.

To a certain extent it appears safe to conclude that as the amount of substance of unknown antirachitic vitamin content is increased the optimum is reached when no further response is obtained with irradiation. This, however, has its limitations because vitamin A must be present in sufficient amounts to allow

normal growth and also because with the introduction of large amounts of such a material as alfalfa the inorganic balance is

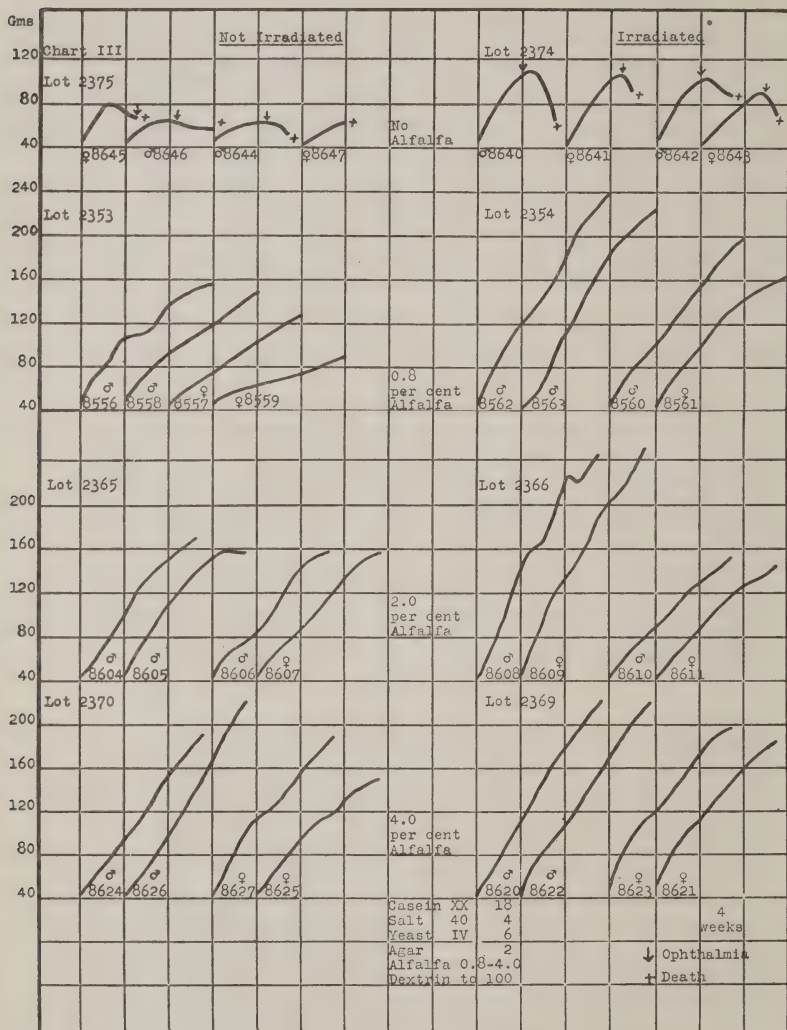


CHART III.

changed so that ultimately the amount of vitamin required may actually be much less, and with other materials may be greater.

Chart IV brings out the importance of a sufficiency of vitamin A in this connection. Some years ago (5) in studying the stability of the fat-soluble complex then spoken of as the fat-soluble vitamin we had occasion to observe that Swiss chard, when kept exposed to light in the attic, gradually lost its ability to support growth in our rats on a dextrin, wheat germ, salt, agar diet. This opened up the matter of the relation of the curing process to the preserva-

TABLE III.

Percentage of Ash in Bones of Rats on Alfalfa-Containing Rations.

Ration.	Lot No.	Rat No.	Sex.	On ration. wks.	Not irradiated.		Irradiated.	
					Humerus.	Femur.	Humerus.	Femur.
0.5 per cent alfalfa.	2349	8540	♂	12	54.8	56.0		
		8541	♀		51.8	52.6		
	2350	8544	♂	12			61.4	61.5
		8545	♀					65.0
0.8 per cent alfalfa.	2353	8557	♀	12.5	60.2	59.0		
		8558	♂		57.4	59.2		
	2354	8561	♀	12.5			61.0	
		8563	♂				62.4	63.4
2.0 per cent alfalfa.	2365	8605	♀	12	63.6	61.8		
		8606	♂		59.5	59.8		
	2366	8609	♀	12			61.5	61.5
		8610	♂				62.0	61.9
4.0 per cent alfalfa.	2369	8621	♀	12	66.2	64.5		
		8622	♂		62.9	62.9		
	2370	8624	♂	12			64.7	66.7
		8625	♀				64.3	63.5

tion of vitamins in our roughages which has had some interesting developments (6). It was from this point of view that the data shown in Chart IV were accumulated.

In these experiments rats from the same litters in part as those used in the other alfalfa experiments and similarly distributed in the various groups were fed the same alfalfa with the exception that this alfalfa had been exposed to the weather (sun, dew, and rain) for 10 days in the field.

The results obtained are seen to be entirely different. The incidence of ophthalmia in irradiated as well as non-irradiated animals suggests an inhibition of growth due to deficiency of vitamin A, making it impossible to determine to what extent the antirachitic vitamin was present. But at the same time there was obtained more than the expected amount of growth with the non-irradiated animals. This suggests an antirachitic activation by sunlight as has been reported by Steenbock and Black (7) for

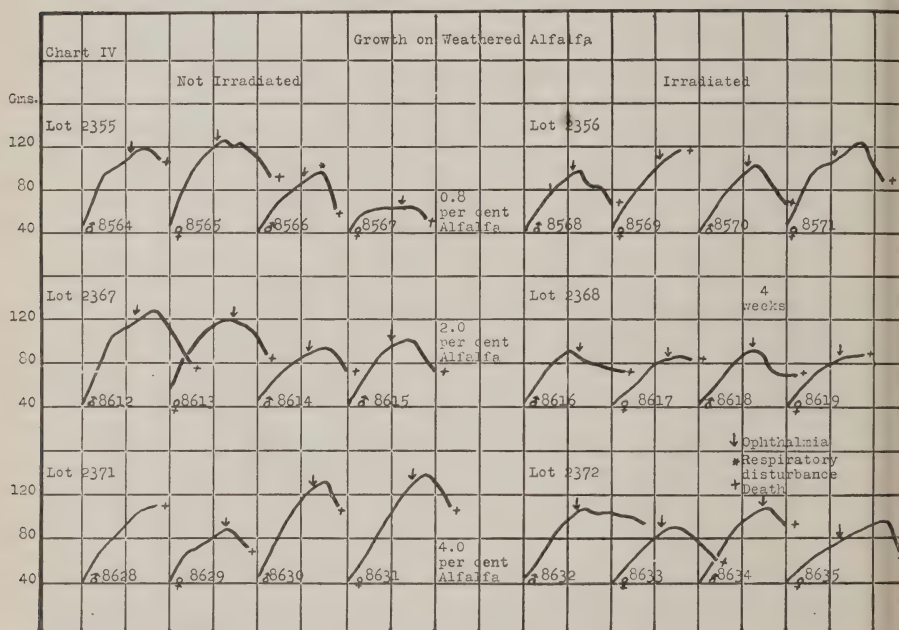


CHART IV.

various rations with the ultra-violet rays of the quartz mercury vapor lamp. Its relation to the antirachitic potency of foods merits further study.

With the above points in mind many experiments on the vitamin A and antirachitic vitamin content of various grains have already been completed. This brief presentation is published with the hope that it will lead to a revision of the growth method hitherto in use for the determination of vitamin A which apparently our preliminary paper of last year (8) failed to effect.

It is, however, probably too optimistic to expect that a growth method in any case is as specific as the ophthalmic method. Growth in its ensemble is too inclusive a reaction, presenting the consummate end-results of physiologic and pathologic reactions. On the other hand, the ophthalmic method experimentally presents the difficulty that ophthalmias are not always incident with vitamin A deficiency. Frequently the infections of the respiratory tract gain the ascendancy and terminate the animal's existence before an ocular reaction sets in. Undoubtedly much is to be gained by the use of the two methods, side by side, in experimental work until they can be correctly evaluated.

SUMMARY.

Absence of growth on suitably constituted rations as used at present cannot be taken as an indication of the absence of vitamin A unless the antirachitic factor is supplied. Recognizing this principle there has been devised a method in which light is used as the antirachitic agent for vitamin A determination. When vitamin A is present in sufficient amounts the amount of antirachitic vitamin can also be determined in foods, provided that the inorganic relations are not seriously disturbed.

It has been concluded that hog millet and probably common millet as well contain considerable quantities of vitamin A. Both, however, are deficient in the antirachitic factor, which is responsible for their failure to support growth when supplemented with casein and our ordinary salt mixture.

Alfalfa carefully cured in the dark was found very rich in vitamin A. Even 0.5 per cent supported normal growth when the antirachitic factor was supplied in the form of light, otherwise 4.0 per cent was required. When cured in the sun, exposed to dew and rain, vitamin A was destroyed but an antirachitic activation of certain substances in the alfalfa is suggested.

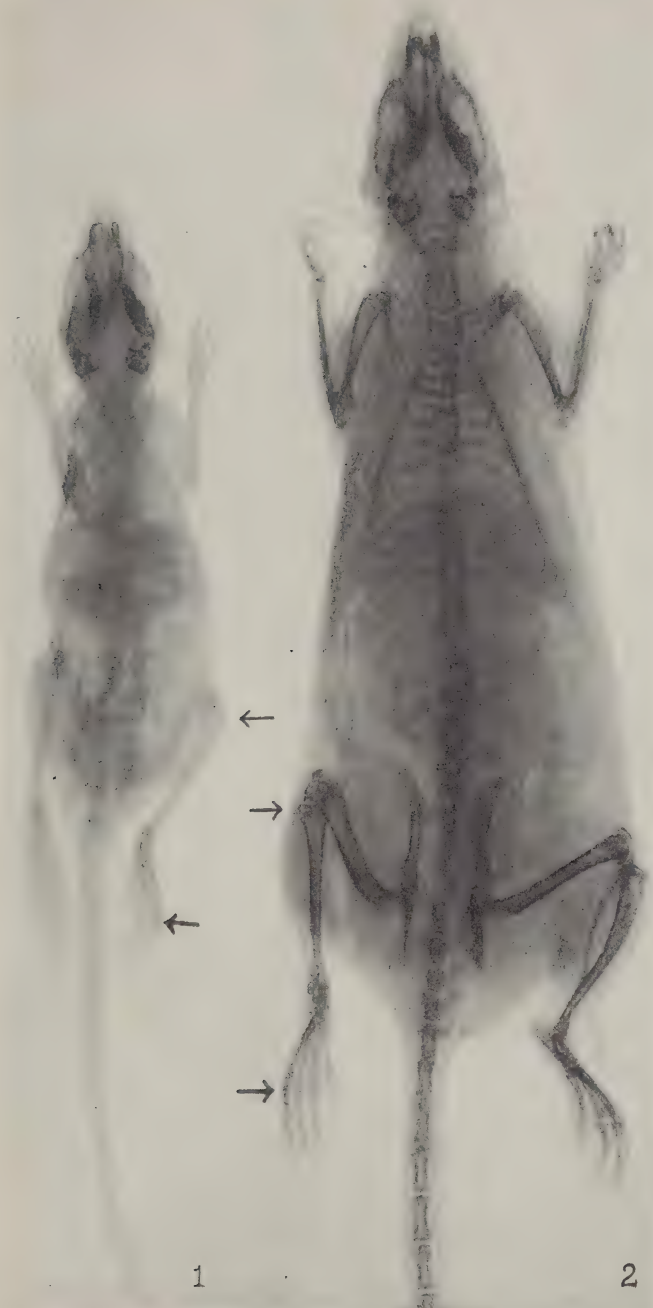
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8. Steenbock, H., Nelson, M. T., and Black, A., *J. Biol. Chem.*, 1924, lix, p. ix.

EXPLANATION OF PLATE 2.

Plate 2 shows radiographs of two female rats both started at 47 gm. and 3 weeks of age on a hog millet ration. Rat 7742, Fig. 2, had been exposed to ultra-violet light while Rat 7732, Fig. 1, had not. Attention is called especially to the difference in size and the rachitic metaphyses of the non-irradiated animal. Rat 7732 weighed 57 gm. and Rat 7742, 220 gm.



(Steenbock, Nelson, and Black: Fat-soluble vitamins. XX.)

THE ESTIMATION OF SUGAR IN DIABETIC URINE, USING DINITROSALICYLIC ACID.

By JAMES B. SUMNER.

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(Received for publication, July 23, 1924.)

Dinitrosalicylic acid was introduced by the author in 1921 as a reagent for the estimation of sugar in normal and diabetic urine.¹ It has also been found to be a valuable reagent for the detection of albumin in urine and has been used for the determination of the titratable alkali of the blood.²

During the past 3 years several improvements have been made in the method for the determination of sugar in diabetic urine. One of the chief errors in copper titration methods is the reoxidation of the cuprous oxide by atmospheric oxygen. With dinitrosalicylic acid, on the contrary, the amount of exposed surface has almost no effect, the same amount of reduction being given with tubes of all diameters. But with dinitrosalicylic acid the oxygen that is dissolved in the solution, while largely incapable of reoxidizing the reduction product, is able to destroy part of the sugar. It has been found that the addition to the reagent of a considerable amount of Rochelle salt largely prevents it from dissolving oxygen and greatly increases the amount of color given by small amounts of glucose. As used in the present modified manner, the amount of color given by 1 mg. of glucose is increased by about 65 per cent. As originally used, dinitrosalicylic acid gave practically no color with as little as 0.1 mg. of glucose, as nearly all of the glucose was oxidized by the dissolved oxygen. With the present modification the reaction is very dependable and the color values show such a good proportionality that the color given

¹ Sumner, J. B., *J. Biol. Chem.*, 1921, xlvii, 5.

² Sumner, J. B., and Hubbard, R. S., *J. Biol. Chem.*, 1923, lvi, 701.

Permanent Standards for Approximate Comparison.—Prepare a solution containing 0.8625 per cent of iron alum. To ten 100 cc. volumetric flasks add 10 cc. of 1 per cent dinitrosalicylic acid and then 1.75, 2.53, 3.22, 4.00, 4.68, 5.32, 6.1, 6.8, 7.5, and 10.3 cc. of the iron alum solution. Dilute to 100 cc. volume and mix. Place 25 cc. of each solution in a Folin-Wu sugar tube of Pyrex glass and stopper with paraffined cork stoppers. These solutions correspond to 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 3.0 mg. of glucose in the order given above.

Method.

Pipette into a Folin-Wu sugar tube 1 cc. of urine (diluted if necessary) and 3 cc. of the reagent. Mix and heat 5 minutes in boiling water. Cool 3 minutes in running water, dilute to 25 cc. volume, mix, and compare in colorimeter with permanent standard set at 20 mm., or with standard prepared with 1 mg. of glucose.

THE OCCURRENCE OF PLANT NUCLEOTIDES IN ANIMAL TISSUES.

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(Received for publication, October 14, 1924.)

A clear distinction has come to be drawn between animal nucleotides and plant nucleotides concerning the carbohydrate group which they respectively contain. The animal nucleotides upon acid hydrolysis produce both levulinic acid and formic acid which are destruction products of hexose and not of pentose. For this reason the animal nucleotides have been assumed to contain a hexose group of unstable character which easily decomposes when produced by such hydrolytic processes as are required for its liberation from the nucleotides.

On the other hand the plant nucleotides possess a pentose group and a pentose is actually formed from some of them by hydrolysis.

In spite of this distinction, two somewhat puzzling matters have for a long time commanded attention; one is the wide distribution in animal tissues and the occasional occurrence in the urine of substances that respond to the qualitative color tests for pentose, a matter that can be provisionally disposed of by assuming that the pentose distributed throughout the animal organism is probably not identical with the one formed by hydrolysis of plant nucleotides.

The second matter cannot be so easily dismissed. Two nucleotides of undoubted plant relationship can be isolated from animal tissues. One is the inosinic acid constantly present in muscle and the other is the guanylic acid prepared from the β -nucleoprotein of the pancreas and other glands. Each of these nucleotides contains not only a pentose group, but on hydrolysis produces the particular pentose (*d*-ribose) that is similarly formed from yeast nucleic acid. When it is recalled that guanylic acid has been

prepared from yeast nucleic acid and found identical in every respect with the substance obtainable from the pancreas, the distinction between animals and plants so far as concerns their nucleotides becomes a little disturbed. We were formerly inclined to believe that the presence of plant nucleotides in animal tissues is caused by the plant food which the animal consumes. But the tentative and confessedly inadequate evidence upon which this view was based has since been found erroneous.

In the present paper it will be shown that the β -nucleoprotein of the pancreas yields not only guanine nucleotide but also cytosine nucleotide and adenine nucleotide, in spite of the assumption made by various experimenters that this nucleoprotein does not contain an adenine group. The results obtained scarcely admit of dispute. The substances isolated possess the crystalline form, chemical composition, and all of the many characteristic properties of the corresponding crystalline nucleotides which have been obtained from yeast nucleic acid. Since uracil nucleotide as a primary nucleotide of plant nucleic acid is somewhat doubtful it would appear that all nucleotides of yeast nucleic acid can be prepared from animal pancreas.

Moreover, substances identical with or nearly related to the β -nucleoprotein of the pancreas have been isolated from many different glands. Those obtained from spleen and liver have been closely examined and from them a substance has been prepared which is apparently identical with the guanylic acid (guanine nucleotide) of the pancreas. It would be strange indeed if they did not also contain adenine nucleotide and cytosine nucleotide. It thus seems more than probable that the distinction between animal and plant nucleic acid will in the future not be so definitely drawn.

Of the greatest interest in this connection is the recent work of Jackson¹ who succeeded in preparing from blood a substance having the general properties of a plant nucleotide. By acid hydrolysis it produces adenine but not guanine and by hydrolysis with ammonia under pressure, phosphoric acid is split off leaving the adenine group still combined. These and other matters led Jackson correctly to the conclusion that he could bring a more certain proof of the presence of adenine nucleotide in blood only by the isolation of the substance itself.

¹ Jackson, H., Jr., *J. Biol. Chem.*, 1923, lvii, 121; 1924, lix, 529.

EXPERIMENTAL.

Preparation of β -Nucleoprotein from the Pancreas.

The following method of preparation might be simply disposed of by a reference to the article of Steudel and Brigl² on the same subject for we have employed a process which is essentially the modification of Bang's method³ which Steudel describes. But we state our results somewhat exactly because of the remarkable difference in the yield which Steudel obtained from that which we obtained with the pancreas of both pig and beef. The phosphorus percentage of Steudel's preparation is also considerably different from that of ours.

Trimmed and ground pancreas whether of beef or pig was thoroughly mixed by stirring with two-thirds of its weight of cold water and after digesting for an hour, with occasional stirring, the material was brought to the boiling point, kept boiling for 10 minutes, and finally allowed to cool thoroughly either by standing overnight at the room temperature or by exposure to the outside freezing temperature for an hour or more. After mechanical removal of the solidified fat the liquid was strained tightly through cloth and the cloudy fluid was then filtered through paper. This filtration is quite slow but continuous and can be allowed to proceed overnight without fear of putrefaction. The fluid is pale yellow and practically water-clear, especially if the runnings of the first 10 minutes are refiltered.

The pancreas residue was ground up a second time with one-third of the volume of water used for the original extraction and the mixture was allowed to stand with occasional grinding and stirring for an hour, when the fluid was strained by pressing through cloth and afterwards filtered through paper as was done with the original extract. A second extraction of the pancreas with water was made in the same way.

The united clear fluids were treated with glacial acetic acid in such an amount as to produce the maximum turbidity (10 cc. per liter of fluid) when a gelatinous, cloudy precipitate was produced which is incapable of filtration and has no tendency to settle even on standing overnight; but upon the addition of an equal volume of 95 per cent alcohol the precipitate becomes flocculent, well defined, and settles sharply, leaving a somewhat cloudy supernatant fluid. After the precipitate had settled overnight the fluid was decanted and the precipitate washed twice by decantation with 95 per cent alcohol. The β -nucleoprotein was then filtered on a Büchner funnel, washed by removal and grinding with absolute alcohol, and finally

² Steudel, H., and Brigl, P., *Z. physiol. Chem.*, 1910, lxxiii, 40.

³ Bang, I., *Z. physiol. Chem.*, 1898-99, xxvi, 133; 1900-01, xxxi, 411.

placed in a desiccator. It is of advantage to remove the partially dried cake from the desiccator after 24 hours and expose it to the air until it loses no more weight. In Table I are the yields obtained by this process from the pancreas of both pig and beef.

TABLE I.

Moist pancreas used.	β -Nucleoprotein obtained.	Per kilo of tissue.
Fig.		
<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1,378	5.6	4.07
5,312	24.0	4.36
7,144	30.5	4.27
2,187	12.4	5.9
2,627	16.0	6.66
2,407	13.8	5.73
2,547	15.4	6.05
2,587	14.2	5.45
Beef.		
5,691	40.3 (P = 3.74)	7.08
3,885	29.4	7.57
3,185	24.5	7.69
2,447	21.0	8.58
2,107	16.0	7.59
3,047	27.4	8.99
3,485	31.9	9.15
2,177	16.7	7.67
3,586	35.7	9.99
4,486	44.0	9.88
3,986	36.9	9.25
3,406	29.0	8.51
3,668	30.7	8.36
3,378	30.0	8.88
By Steudel.		
<i>kg.</i>		
25.5	500.0 (P = 4.45) 4.62)	20.0

The numbers given in Table I show that while a somewhat greater amount of material is obtainable from the pancreas of the beef than that of the pig, this difference, formerly supposed to be very significant, is not nearly so great as two different experimenters may obtain from glands of the same species.

Preparation of Crude Nucleotides from β -Nucleoprotein of the Pancreas.

When the work described below was undertaken we knew rather definitely that other nucleotides than guanine nucleotide could be prepared from the β -nucleoprotein, but we did not know whether these substances were present as mononucleotides easily capable of manipulation and identification or in the form of compound nucleotides whose manipulation would be difficult and whose identification would be practically impossible. Mononucleotides are characteristic, capable of separation from one another and subsequent identification; whereas compound nucleotides (as nucleic acid) have no characteristics by which they can be identified nor do they lend themselves to analytical processes. For these reasons the β -nucleoprotein of the pancreas was treated initially in such a way as to insure the conversion of all compound nucleotides present into mononucleotides. This consisted in digesting the material at the room temperature with a dilute solution of sodium hydroxide. Under these conditions, as has been pointed out, any compound nucleotides present will be decomposed quantitatively into their constituent mononucleotides, but the latter will not suffer further decomposition, either by hydrolysis or deamination, in any reasonable length of time.

Accordingly, portions of the β -nucleoprotein (12 gm.) were suspended in water (175 cc.) and enough 20 per cent sodium hydroxide was added (15 cc.) to make the solution 1.25 per cent. The product was allowed to digest at the room temperature overnight when flocculent phosphates which had settled sharply were filtered off and after faint acidification with acetic acid the warm fluid was treated with hot neutral lead acetate for the precipitation of the nucleotides. The lead precipitate was filtered, washed by grinding with warm water, suspended in hot water, and decomposed with sulfurated hydrogen. After filtering off the lead sulfide and aeration the fluid was evaporated under diminished pressure at 40°, precipitated, hardened with absolute alcohol, and finally allowed to dry in a desiccator.

This material was produced in about equal quantities from the nucleoprotein of pig and beef. It gave a very faint biuret reaction but remarkable color tests for pentose with both phloroglucin and orcin. It could easily be shown to produce on hydrolysis phosphoric acid, guanine, and adenine. Its phosphorus was in part easily split and in part firmly bound. No difference could be shown between the preparations obtained from the two animal species.

Separation of the Mixed Nucleotides into Two Fractions.

This separation was formerly accomplished by treating a solution of the mixed nucleotides in ammonia with an equal volume of absolute alcohol. Under these conditions the ammonium salt of guanine nucleotide is precipitated while the other nucleotides remain in solution. This method, however, has been replaced by one based on the following principle.

When guanine nucleotide is purified by alternate solution in potassium hydroxide and precipitation with acetic acid the substance (which is evidently a potassium salt) continually loses potassium so that after a few reprecipitations the faintly alkaline solution fails to give any precipitate at all with acetic acid even after the addition of alcohol; but upon the addition of a trace of concentrated aqueous potassium acetate a precipitate immediately appears.

A portion of guanine nucleotide was dissolved in warm water and the solution made faintly alkaline with potassium hydroxide. On cooling, the solution gelatinized, but on warming and diluting with warm water the gelatinous material passed easily into permanent solution. After cooling, this solution was treated with a few drops of concentrated aqueous potassium acetate when a dense stringy precipitate was thrown out which upon the addition of an equal volume of alcohol separated sharply, leaving a perfectly clear, easily filterable interstitial liquid.

On the other hand when adenine nucleotide is brought into dilute solution with a trace of potassium hydroxide the addition of potassium acetate forms no precipitate whatever nor does the subsequent addition of an equal volume of alcohol produce the faintest cloud.

Upon the facts stated, the following method of separating the mixed nucleotides from one another was based.

Portions of the mixed nucleotides (about 15 gm.) were dissolved in hot water (125 cc.) in which the nucleotides are easily soluble forming a pale yellow, perfectly transparent solution. A trace of aqueous potassium acetate was then added (1 cc. per 1 gm. of nucleotide mixture). This threw out the stringy precipitate which was increased in amount and became flocculent upon the addition of an equal volume of 95 per cent alcohol. After standing overnight in a corked vessel the precipitated guanine nucleotide was filtered off, dissolved in hot water with a little sodium hydroxide, precipitated again with potassium acetate and alcohol, and filtered sharply on a Büchner funnel.

I. The Guanine Fraction.

The cake consisting of the potassium salt of guanine nucleotide is of no interest in this connection.

II. The Adenine Fraction.

The united filtrates from guanine nucleotide were made faintly acid with acetic acid, treated with an equal volume of boiling water and then with neutral lead acetate for the precipitation of the lead salts of all nucleotides present. The lead precipitate after thorough washing was suspended in water and decomposed with sulfured hydrogen. The concentrated solution obtained by evaporating the aerated filtrate from lead sulfide at 40° under diminished pressure was allowed to stand in a cool place overnight when beautiful stout individual transparent prisms were deposited. These were filtered off, recrystallized from hot water and analyzed.

0.2882 gm. required 10.37 cc. standard acid (1 cc. = 0.003547 N).

0.3382 " gave 0.2485 gm. $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$.

	Calculated for cytosine nucleotide.	Found.	
		I	II
N.....	13.00	12.83	
P.....	9.59		9.29

The nucleotides present in the filtrate from this crystalline nucleotide were precipitated and hardened with absolute alcohol. The preparation should contain three of the reputed plant nucleotides (adenine nucleotide, cytosine nucleotide, and uracil nucleotide) if they were originally present in the crude nucleotide mixture. The material is referred to below as the nucleotides of the adenine fraction.

5 gm. were hydrolyzed for 2 hours with 20 parts of 7 per cent sulfuric acid. The resulting solution which was only slightly darkened was treated with ammonia for the precipitation of any possible traces of guanine. At first no precipitate occurred, but on standing overnight a flocculent precipitate was produced. The quantity was not sufficient for identification, but the substance was found to consist mostly of iron phosphate. The filtrate from this precipitate was treated with magnesia mixture for the removal of phosphoric acid and then with a solution of silver nitrate in ammonia. The bulky, transparent, gelatinous precipitate thus produced was suspended in water, decomposed with sulfured hydrogen, and evaporated to dryness on the water bath several times after moistening with water for the expulsion of all free hydrochloric acid. The residual adenine chloride was

converted into adenine sulfate by recrystallization from hot 5 per cent sulfuric acid. Colorless, transparent, macroscopic crystals were obtained. A solution of the material in warm water produced with picric acid the characteristic pale yellow precipitate of adenine picrate, consisting of long, intertwined needles resembling matted hair.

0.2433 gm. lost 0.0219 gm. at 125°.

0.1047 " (0.1151 gm. hydrous) required 11.24 cc. standard acid.

0.1165 " (0.1280 " ") " 12.42 " " "

	Required for adenine sulfate.	Found.		
		I	II	III
2H ₂ O	8.91	9.00		
N	34.65		34.63	34.41

The Isolation of Crystalline Adenine Nucleotide and Crystalline Cytosine Nucleotide by Means of Their Brucine Salts.

30 gm. of the nucleotides of the adenine fraction were converted into brucine salts by treatment of an aqueous solution of the nucleotides with an alcoholic solution of brucine, nearly to the neutral point. The precipitated brucine salt mixture after thorough washing was crystallized nine times out of 35 per cent alcohol and an examination was made of the initial brucine salt, the final crystalline product, and also the crystalline product obtained by allowing the various mother liquors to evaporate to half their volume at the room temperature.

I. The Brucine Salt Initially Obtained.

A portion of this material, after removal of the brucine, was used for the preparation of the free nucleotides in the usual way through the lead salts. After evaporation of the final filtrate from lead sulfide under diminished pressure at 40°, a concentrated solution of the nucleotides was finally obtained which gave only a slight deposition on cooling, but after standing for several days gradually deposited in profuse amount crystalline needle clusters having the characteristic appearance of adenine nucleotide. By hydrolysis with 20 parts of 7 per cent sulfuric acid this product produced no guanine, gave a large precipitate of magnesium ammonium phosphate when treated with magnesia mixture, and produced with

silver nitrate and ammonia the characteristic transparent gelatinous precipitate of silver-adenine. The material lost its water of crystallization by heating in an air bath, but regained it when exposed to air.

A small portion of this material upon recrystallization from hot water exhibited supersaturation to a remarkable degree, but was finally deposited in beautiful needle clusters.

0.1817 gm. required 9.96 cc. standard acid.

	Required for adenine nucleotide.	Found.
N	19.18	19.44

II. The Brucine Salt Obtained by Evaporation of the Mother Liquors from the First, Second, Third, and Fourth Recrystallizations.

This material, after removal of the brucine, was used for the isolation of free nucleotides through their lead salts as described. A beautifully crystalline product having the appearance and properties of crystalline adenine nucleotide was finally obtained.

0.1830 gm. required 9.90 cc. standard acid.

	Required for adenine nucleotide.	Found.
N	19.18	19.18

III. The Final Product Obtained After Recrystallization Nine Times from 35 Per Cent Alcohol.

I. 0.5380 gm. required 12.10 cc. standard acid.

II. 0.4714 " " 10.62 " " "

III. 0.4597 " " 10.33 " " "

	Required for brucine salt of:		Found.		
	Uracil nucleotide.	Cytosine nucleotide.	I	II	III
N	6.79	7.92	7.97	7.99	7.97

From this substance the free nucleotide was prepared in the usual way. Cytosine nucleotide, consisting of beautiful transparent plates, was finally obtained. On hydrolysis it produced no trace of any purine compound precipitable by silver nitrate and ammonia. On heating at 105° it lost no weight.

I. 0.2392 gm. required 8.78 cc. standard acid.

II. 0.3017 " " 11.10 " " "

III. 0.3221 " gave 0.2445 gm. $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$.

	Required for cytosine nucleotide.	Found.		
		I	II	III
N.....	13.0	13.01	13.05	
P.....	9.59			9.60

ANTIRACHITIC PROPERTIES IMPARTED TO INERT FLUIDS AND TO GREEN VEGETABLES BY ULTRA-VIOLET IRRADIATION.

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Columbia University, New York.)

(Received for publication, September 16, 1924.)

In June of this year we made a preliminary report (1) to the effect that, by means of irradiation, vegetable oils can be endowed with antirachitic properties.¹ During the past few months this investigation has been extended and has yielded further interesting results.

It has been shown by numerous investigators that under certain conditions growth can be brought about in young rats by subjecting them to the rays of the mercury vapor lamp. Somewhat over a year ago Hume and Smith (2) reported that they were able to stimulate growth by keeping rats in an atmosphere which had been irradiated with the ultra-violet rays produced by this lamp. These experiments have not been corroborated; in fact, Webster and Hill (3), in a paper treating of "The supposed influence of irradiated air on growth" recently reported negative results. About 2 years ago we attempted to endow air with antirachitic properties by means of this form of irradiation. Two experiments of this nature were carried out. In the first the air in an 8 liter jar was irradiated for 15 minutes at a distance of 1 foot. Young rats, weighing about 40 gm., were then promptly placed in the jar and were kept there for a period of 90 minutes; after this period the air was again irradiated and the animals put back in the jar. The rats, which were on the standard low phosphorus

¹ Since this preliminary report Steenbock has communicated results which seem to confirm our work. In a recent issue of *Science* (Sept. 5, 1924) he states that by "irradiating fats" he succeeded in rendering them active in preventing rickets.

diet (Sherman-Pappenheimer), developed rickets to about the same degree as those which had not been exposed to the irradiated air (Table I). The experiment was repeated, placing the lamp at half the distance (6 inches) and subjecting the animals to three daily periods of $1\frac{1}{2}$ hours each. The jar was lined with paper so as to preclude the possible action of the rays on the glass, and calcium chloride was placed in the jar to absorb the moisture. Once more the result was negative as will be noted in Table I.

TABLE I.
Attempt to Prevent Rickets by Exposing Rats to Irradiated Air.

Rat No.	Weight. <i>gm.</i>	Rickets- producing diet.	Air.	Rickets.	
				Radiographic.	Microscopic.
5125	54-52	Low phos- phorus. No. 84.	Interior of jar irradiated twice for 15 min. at a distance of 1 ft. Rats exposed for a total of 3 hrs.	Moderate.	Moderate.
5128	36-34			Slight.	Slight.
5129	30-42			Moderate.	Moderate.
5126	54-50	" "	Non-irradiated.	"	"
5127	40-34			"	"
5235	36-36	" "	Interior of jar irradiated three times for 15 min. at a distance of 6 in. Rats exposed for a total of $4\frac{1}{2}$ hrs.	Marked.	Marked.
5236	30-40			"	"
5237	20-30			"	"
5238	24-38			"	"
5239	24-30	" "		"	"
5240	30-36			"	"
5241	22-38			"	"
5242	24-36			"	"

Distilled water was similarly irradiated with the object of developing the antirachitic factor. For this purpose the lamp was placed at a distance of 1 foot and the irradiation carried out for 1 hour immediately previous to feeding the water to the rats. Three experiments of this kind were performed, the animals receiving from 0.5 to 1.0 cc. of the irradiated water daily by pipette. The control rats received untreated distilled water in addition to the low phosphorus diet. In one of these experiments

the irradiated water seemed to possess some antirachitic potency, in the two others there was little or no distinction in the develop-

TABLE II.

Attempt to Prevent Rickets by Feeding Rats Water Which Had Been Irradiated or Ozonized.

Rat No.	Weight. <i>gm.</i>	Rickets-producing diet.	Substance fed.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
6304	40-40	Low phosphorus. No. 84.	0.5 cc. distilled water, irradiated 1 hr. at 1 ft.	No(?).	No.	Very sl.
6305	34-44			"	"	" "
6306	38-40			Slight.	Very sl.	Slight.
6307	30-30			No(?).	No.	"
6576	32-34	" "	0.5 cc. distilled water, irradiated 2 hrs. at 1 ft.	No.	Very sl.	"
6577	30-34			Slight.	" "	
6578	60-64			"	Slight.	Moderate.
6579	50-50			Very sl.	"	Negative.
6580	40-41			Marked.	Moderate.	
6409	70-82	" "	1 cc. distilled water, irradiated 1 hr. at 1 ft.	Moderate.	Marked.	Moderate.
6410	60-52			"	"	"
6411	54-56			Marked.	"	"
6433	44-46	" "	0.5 cc. distilled water, ozonized $\frac{1}{2}$ hr.	Moderate.	Moderate.	Marked.
6434	46-48			Slight.	Slight.	Moderate.
6435	62-63			"	"	Slight.
6436	60-66			"	"	"
6412	70-69	" "	1 cc. distilled water, ozonized 1 hr.	Marked.	Marked.	Marked.
6413	50-50			Moderate.	"	Moderate.
6414	54-56			"	Moderate.	"
6441	60-68	" "	0.5 cc. distilled water.	"	"	"
6442	42-44			"	"	"
6415	70-72	" "	1 cc. distilled water.	Marked.	Marked.	Marked.
6416	58-60			Moderate.	Moderate.	Moderate.
6417	68-70			Marked.	Marked.	Marked.

ment of rickets between the rats which had received the untreated or the irradiated water (Table II). These results will have to be

interpreted in the light of further experiments. After the favorable test with water several experiments were undertaken with

TABLE III.
Attempt to Prevent Rickets by Giving Rats Radium by Mouth or Subcutaneously.

Rat No.	Weight.	Rickets-producing diet.	Amount of radium given.	Route radium was given.	Result in regard to rickets.	Blood P (inorganic).
	<i>gm.</i>					<i>mg. per cent</i>
4001	40-46	Low phosphorus. No. 84.	1/160 mkg. Ra bromide, water 0.25 cc.	Subcutaneous.	Marked.	2.53
4002	50-70				"	
4003	40-44	" "	1/80 mkg. Ra bromide, water 0.5 cc.	"	"	
4004	40-50	" "	1/40 mkg. Ra bromide, water 1.0 cc.	"	"	2.19
4005	42-50				"	
6463	40-40	" "	0.5 cc. of 2 per cent radium water.	Oral.	"	
6464	40-40				"	
6466	32-30				"	
6467	30-28				Moderate.	
6548	80-75	" "	0.25 cc. of 20 mkg. Ra bromide in 87 cc. linseed oil.	"	"	
6549	40-43				"	
6550	50-60				"	
6551	50-48				"	
6552	44-46	" "	0.25 cc. linseed oil.	"	Slight.	
6553	80-80				Moderate.	
6483	50-52	" "			Marked.	
6484	70-60				Moderate.	
6485	58-54				Marked.	
6554	80-80				Moderate.	
6555	50-54				"	

the object of explaining the action of the rays. It was thought that ozone might play a rôle, for it is well known that ozone is

produced by the mercury vapor lamp. To test this hypothesis water was subjected to an electric ozonizer. This ozonized, water was fed to the rats, but without effect. Somewhat later, ozonized cottonseed oil was employed, but the result was likewise negative. In view of the fact that it has been stated that ultra-violet rays form hydrogen peroxide from water, a feeding experiment with

TABLE IV.

Prevention of Rickets by Feeding Rats Cottonseed Oil Which Had Been Irradiated Immediately or 20 Days Previously.

Rat No.	Weight.	Rickets-producing diet.	Cottonseed oil.	Rickets.			Blood P. (inorganic).
				Radio-graphic.	Macroscopic.	Microscopic.	
	<i>gm.</i>						<i>mg. per cent</i>
6353	40-50	Low phosphorus. No. 84.	0.1 cc. irradiated 1 hr. at 1 ft.	No.	No.	Negative.	6.21
6354	40-40			"	"	"	
6357	30-30			"	"	"	
6378	44-52	" "	0.1 cc. irradiated 1 hr. at 1 ft. Stored 20 days.	"	"	"	
6379	36-34			"	"	"	
6381	34-30			"	"	"	
6382	28-28			"	"	"	
6358	48-46	" "	0.1 cc.	"	"	"	2.13
6360	34-40			Marked.	Moderate.	Marked.	
6361	30-30			Moderate.	Marked.	"	
6362	44-50			Slight.	Slight.	Slight.	
6384	40-50	" "		Moderate.	Moderate.	Moderate.	1.79
6385	44-41			Marked.	Marked.	Marked.	
6386	28-36			"	"	"	

this substance was carried out. 0.1 cc. of a 0.25 and of a 0.5 per cent solution of peroxide was given; the solution was made up in water from perhydrol. However, hydrogen peroxide proved to have no protective value.

The effect of radium was also tested. A 2 per cent solution of radium bromide in water was given orally and also subcutaneously.

Table III shows the failure of these experiments. A similar negative result was obtained with radium bromide suspended in linseed oil;² 0.25 cc. was given daily to each rat.

Our first definitely favorable result was obtained when we used irradiated cottonseed oil. A large series of experiments with this product was carried out. The oil was prepared by placing it in Petri dishes in layers so thin that they barely covered the surface of the dish. In fact it was found necessary to agitate the dishes in the course of the irradiation in order to keep their surfaces entirely covered and to prevent the oil from drying. As mentioned in our preliminary communication the oil acquired a peculiar fishy odor after treatment, an odor suggestive of cod liver oil. The mercury vapor lamp was placed at a distance of a foot, the irradiation was always continued for 1 hour, and the oil was fed to the rats by pipette immediately thereafter. The control rats received the same amounts of untreated oil—0.1, 0.25, or 0.5 cc. daily. It was found that the irradiated oil protected the rats in spite of the severity of the rickets-producing dietary. 0.1 cc. was sufficient to exert this prophylactic effect (Table IV). In order to ascertain whether the oil retained its acquired antirachitic power, cottonseed oil was irradiated and kept in the dark, covered with paraffin for a period of 20 days. So far as could be determined it had not lost its activity—it was able to confer protection when given in the dosage of 0.1 cc. daily. Tests with oil which was irradiated some months ago are now in progress. In the course of our experiments, as we increased the dosage of cottonseed oil, it was found that large amounts (0.5 cc.) possess slight antirachitic properties. However, 0.1 cc. was insufficient to afford protection.

Other oils were similarly tested. Linseed oil was first selected as it is supposed to possess the least antirachitic action. An extended series of experiments with this oil showed that following irradiation it likewise protected rats from rickets (Table V). It will be noted in this table that the rats which were protected by this means had a comparatively high percentage of inorganic phosphorus in the blood, 6.84 mg. per cent, as compared with 3.22 mg. per cent in the controls which received the untreated oil,

² This suspension was prepared at the laboratory of the United States Radium Corporation.

TABLE V.
Prevention of Rickets by Feeding Rats Irradiated Linseed Oil.

Rat No.	Weight.	Rickets-producing diet.	Linseed oil.	Rickets.		Blood P (inorganic). mg. per cent
				Radiographic.	Microscopic.	
6471	40-34	Low phosphorus. No. 84.	0.1 cc. irradiated 1 hr. at 1 ft.	No.	Negative.	6.84
6472	50-50			"	"	
6473	56-50	" "	0.25 cc. irradiated 1 hr. at 1 ft.	"	"	
6474	30-30			" (?)	"	
6475	40-34			"	"	
6477	38-30			" (?)	"	
6486	40-40	" "	0.25 cc. irradiated 1 hr. at 1 ft. through glass filter.	Moderate.*	Moderate.	
6487	70-64			Marked.*	"	
6488	26-23			" *	Marked.	
6489	30-34			" *	Moderate.	
6478	40-40	" "	0.25 cc.	Moderate.	Marked.	3.22
6479	36-36			"	"	
6480	64-64			Marked.	Moderate.	
6481	44-54			"	"	
6482	34-40			Moderate.	Marked.	
6532	50-50			No.	Negative.	
6533	50-50	" "	0.5 cc. irradiated 1 hr. at 1 ft.	"	"	3.47
6534	70-60			"	"	
6535	44-40			"	"	
6536	40-30			"	"	
6537	54-50	" "	0.5 cc.	"	Slight.	1.72
6538	60-70			Marked.	Moderate.	
6539	64-60			No.	Negative.	
6540	50-54			Moderate.	Moderate.	
6541	50-54			Marked.	Marked.	
6483	50-52			"	"	
6484	70-60	" "		Moderate.	Moderate.	3.33
6485	58-54			Marked.	Marked.	
6528	52-50	" "		Moderate.	Moderate.	3.04
6529	44-44			"	"	
6530	64-54			"	"	
6531	44-41			"	"	

* Signs of healing.

and 3.33 mg. per cent in those which obtained the rickets-producing dietary and no oil. The phosphorus content of the linseed oils which we used was, respectively, 6.81 and 15.62 mg. per 100 cc. It is evident, therefore, that the amount of phosphorus fed to the rats in 0.1 mg. of oil was negligible. It may be added that the phosphorus content of the cottonseed oil was too small to be estimated. The nitrogen content of the linseed oil was 98 mg. per cent, so that the amount fed to the rats was minimal. As the result of irradiation, the oil was found to have been changed but little in the iodine number, that is to say, in its content of unsaturated fatty acid, although the total acidity was very slightly increased.

TABLE VI.

Attempt to Prevent Rickets by Feeding Rats Mineral Oil Which Had Been Irradiated.

Rat No.	Weight. <i>gm.</i>	Rickets-producing diet.	Mineral oil.	Rickets.		
				Radio-graphic.	Macroscopic.	Microscopic.
6348	44-40	Low phosphorus. No. 84.	0.5 cc. irradiated 1 hr. at 1 ft.	Marked.*	Moderate.*	Marked.
6349	50-50			" *	" *	"
6350	50-54			" *	" *	" *
6351	50-48	" "	0.5 cc.	"	Marked.	"
6352	50-46			"	"	"

* Signs of healing.

An attempt was made by means of a selective glass filter to ascertain the length of ultra-violet rays which possess the remarkable alterative effect on oil. For this purpose the Corning filter 586 A was used, the same which we employed some time ago (4) in determining the length of ultra-violet radiations which are effective in rickets. The glass plate (4.3 mm. in diameter) was interposed between the lamp and the film of oil and the total distance reduced from 1 foot to 6 inches. This filter permits the passage of a large intensity of rays of $313\text{ m}\mu$ in length and a small intensity of rays of $302\text{ m}\mu$. It will be seen (Table V) that when this filter was interposed the oil did not acquire protective qualities although the bones of the rats showed signs of healing. This experiment was carried out only once. Further tests will have to

be made to determine the length of the radiations which produce the change in oils.

(Olive oil was tested in one series of rats with an irregular result. It is possible that the irregularity was due to an excessive thickness of the layer of oil which was exposed. This is a danger that should be constantly guarded against, on account of the weak penetrating power of the ultra-violet radiations.

(We next tested mineral oil. Five rats were given 0.5 cc. of this oil after it had been irradiated. They all developed marked rickets, one showing slight healing (Table VI). No diarrhea was occasioned by this oil.

TABLE VII.

Attempt to Prevent Rickets by Feeding Rats Gelatin Which Had Been Irradiated.

Rat No.	Weight. <i>gm.</i>	Rickets- producing diet.	Gelatin.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
6601	30-28	Low phos- phorus. No. 84.	0.25 cc. 1½ per cent irradi- ated 2 hrs. at 1 ft.	Marked.	Marked.	Marked.
6602	34-46			Moderate.	Slight.	
6603	36-34			Slight.	"	Slight.
6604	50-50			Moderate.	Marked.	Moderate.
6605	34-40			"	Moderate.	"
6606	32-40	" "	0.25 cc. 1½ per cent.	"		"
6607	50-50			"	"	"
6608	36-36			Marked.	Marked.	Marked.
6609	30-31			Moderate.	Moderate.	Moderate.
6610	30-34			"	"	

The effect of irradiation on other fluids was also tested biologically. Cream (20 per cent) was rayed for a period of 2 hours at a distance of 1 foot and fed in 0.25 cc. amounts; milk, for 1 hour at 1 foot and was given in 0.5 cc. amounts; a 1.5 per cent solution of gelatin was rayed for 1 hour at the same distance and 0.25 cc. fed daily (Table VII); and human serum was treated in the same way and then given in 0.1 cc. amounts. The results of these various tests were not uniform, thus rendering them difficult of interpretation. The test of human serum was of special interest in view of the employment of ultra-violet irradiation in clinical medicine. Of the six rats fed with the treated serum three developed rickets and three failed to show any lesions.

DISCUSSION.

It is too early to discuss at length the nature of the changes brought about in these fluids which endow them with antirachitic potency; nor can it be definitely stated that it is the same factor which gives to cod liver oil its specificity in rickets. If such prove to be the case, and this factor is to be regarded as a vitamin, then these results constitute the first demonstration of the production of a vitamin *in vitro*. All that can be deduced at present is that they furnish evidence of the production of an antirachitic factor outside of a living organism. The question of the therapeutic value of this procedure is of secondary importance. It would seem of value in times of deprivation or stress, as occurred during the recent war, when cod liver oil was unobtainable in the Central Empires.

Antirachitic Properties Imparted to Lettuce and to Growing Wheat by Ultra-Violet Irradiation.

Having ascertained that inert vegetable substances, such as oils, acquire antirachitic potency by exposure to the radiations of the mercury vapor lamp, it seemed of value to investigate whether a similar effect could be brought about in growing vegetables. Can a similar difference be demonstrated between a vegetable grown in the dark and one which is grown in the light and subjected to the radiations of the mercury vapor lamp? To this end winter wheat was grown in the laboratory in darkness and in light. The soil and all the surrounding circumstances were identical. The wheat grew very rapidly, obtaining a height of 6 inches or more in a period of a week or 10 days. That which was grown in the dark room was etiolated, pale yellow in color, tall, and thin, whereas that grown in the light room and irradiated was deep green and more robust. The irradiation was carried out daily for a period of 1 hour at a distance of 3 feet. Each rat was given 10 gm. of wheat daily and, after the first day or two, consumed his entire quota. The rats were kept in the dark in individual cages and given the standard low phosphorus diet (No. 84). The result of this experiment, which is reproduced in Table VIII, was that the rats receiving the etiolated wheat developed rickets, whereas those which received the green, irradiated wheat were normal in this respect.

The further question arose as to whether growth played a rôle in the production of the antirachitic factor in the irradiated wheat or whether the process was quite independent of growth and comparable to the change brought about by irradiating the cottonseed or the linseed oil. To elucidate this point, we used green

TABLE VIII.

Prevention of Rickets by Feeding Rats Irradiated Lettuce or Growing Wheat.

Rat No.	Weight.	Rickets-producing diet.	Substance fed.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
	<i>gm.</i>					
6630	40-64	Low phosphorus. No. 84.	10 gm. wheat irradiated 1 hr. at 3 ft., daily, while growing.	No(?).	No.	No.
6631	40-50			"	"	"
6632	44-70			"	"	"
6633	40-60	" "	10 gm. wheat (etiolated).	Moderate.*	Moderate.	Moderate.
6634	44-68			Slight.	Slight.	"
6635	40-60			Moderate.*	Moderate.	"
6636	40-59	" "	10 gm. green lettuce irradiated 1 hr. at 1 ft., daily.	No(?).	No.	No.
6637	40-52			"	"	"
6638	40-60			Very sl.	"	Very sl.
6639	24-60	" "	10 gm. green lettuce (non-irradiated).	No(?).	"	No.
6640	41-61			Marked.	Moderate.	Marked.
6641	34-54			"	Marked.	"
6642	40-64	" "	" "	"	Moderate.	"
6643	44-50			Moderate.	"	Moderate.
6644	40-42			"	Marked.	"

* Signs of healing.

lettuce leaves. Some was fed just as it came from market and some, after it had been irradiated for 1 hour at a distance of 1 foot. As in the previous experiment 10 gm. were given daily to each rat, the basal diet being No. 84 and the rats being kept in the dark. The result was similar to that obtained when the wheat was fed—a development of rickets when the untreated lettuce was given,

protection against rickets when the irradiated lettuce was added to the dietary (Table VIII).

Before considering these experiments it will be of advantage to discuss briefly the value of green vegetables in the rickets of infants and animals. In our experience green vegetables are unable to protect infants against rickets. Some years ago, Hess and Unger (5), in considering the rôle of the fat-soluble vitamin in rickets, emphasized the fact that rickets could develop in spite of a diet which included 30 gm. of spinach daily. The same holds true in regard to rats. In this laboratory Zucker has fed young rats spinach *ad libitum* and failed to prevent the development of rickets. An appreciation of the inefficacy or unreliability of green vegetables in rickets is of importance, especially as some investigators maintain that the antirachitic factor and the A (fat-soluble) vitamin occur concurrently in nature, so that from a dietetic standpoint it is not necessary to draw a distinction between them. Such, however, is by no means the case, as is illustrated by the lack of the antirachitic factor in spinach which is exceptionally rich in the A (fat-soluble) vitamin. In view of our experiments with irradiated lettuce the question arises as to whether green vegetables may not vary in their antirachitic potency according to the degree of direct sunlight to which they have been subjected and perhaps according to the interval which has elapsed between the time they are plucked and consumed. Both of these points will be considered in a subsequent communication. The comparative value of vegetables which are grown in the hothouse, where the ultra-violet rays have been filtered out by the glass, is one which also requires experimental investigation. Some years ago this question was investigated in relation to the antiscorbutic vitamin. Tomatoes which had been grown in the field were compared in quantitative feeding experiments on guinea pigs with those which had been raised in a hothouse. It was found that the former were slightly superior to the latter in preventing the development of scurvy. The distinction was by no means as definite or marked as in the case of the antirachitic factor. Our results with the wheat and the lettuce call to mind the interesting investigation of Luce (6) who has recently shown that milk "attains a maximum level of antirachitic potency . . . when the cow was placed under conditions of maximal light" and at the same time was

given a diet of fresh green grass. How much of this effect is to be attributed to the action of the sunlight on the cows and how much to the green fodder remains to be determined.

SUMMARY.

Various inert fluids were irradiated with the mercury vapor lamp in order to ascertain whether by this means they could be endowed with antirachitic potency. It was found that cottonseed oil and linseed oil could be rendered specifically active by this means. After these oils had been irradiated they were able to protect rats from rickets when 0.1 cc. daily was added to the rickets-producing dietary. An antirachitic factor therefore had been produced *in vitro* and outside the living organism. The irradiated oils were able to store this factor for a considerable period.

Wheat which was grown in the dark (etiolated) was found to have no antirachitic potency. Wheat which was grown in the light and irradiated with the mercury vapor lamp conferred protection when fed to rats.

The same difference in regard to protective action against rickets was observed in vegetables which were irradiated after they had been plucked. Green lettuce leaves from the market were of no value in preventing rickets, whereas after irradiation they had become antirachitic. In the plucked as well as in the growing green vegetable, irradiation led to the formation of an antirachitic factor.

In these experiments an antirachitic factor was produced both *in vitro* and in the growing plant.

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NEW OBSERVATIONS AND A REINTERPRETATION OF OLD OBSERVATIONS ON THE NUTRITIVE VALUE OF THE WHEAT PLANT.*

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In 1907, we began our inquiries into the validity of the prevailing theory of what constitutes a balanced ration (1). Protein and energy, with no emphasis upon the mineral make-up of the ration, were the sole recognized factors in nutrition at that time. The lapse of 17 years has disclosed the importance of additional factors in animal nutrition, such as the quality of the proteins, the amount and quality of the mineral make-up of the ration and of the vitamins. In our early experiments showing the inadequacy of the prevailing theory of nutrition, use had been made of growing heifers, and a balanced ration from the wheat plant as well as balanced rations constructed from the corn plant, the oat plant, and a mixture of the three. By "balanced" is meant that we conformed to the requirements of prevailing feeding standards, furnishing our animals with the proper quota and proportions of digestible protein, digestible carbohydrates, and digestible fats (energy) prescribed in such standards. In the light of our present knowledge of nutrition, a brief retrospect of these early observations is of interest.

Contrast of the extremes in effect will only be given. Nutrition was complete on the corn plant ration, while it was a failure on the wheat plant ration. For the first year, there was no great

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difference in the rates of growth of the animals confined to the two rations, although the corn-fed animals looked smoother in coat, fuller through the barrel, and in a good state of nutrition, while the wheat-fed group were rough in coat, gaunt and thin in appearance, small of girth and barrel, and, to the practised eye, in rather a lower state of nutrition. The wheat-fed group, if slightly excited or hurried, would collapse and remain prostrated for a few minutes suffering muscular rigor and tremor. From this condition, the animals would gradually recover and appear normal only after a lapse of 10 to 15 minutes. *Some of these animals became blind.* In this group the estrus cycle was delayed and in some individuals never appeared. Where the estrus cycle did appear and the animal became pregnant, the offspring was prematurely born and was either dead or extremely weak and undersized and died soon after birth. The placental membranes were slow in coming away and retention of the afterbirth resulted, with its attendant dangers of infection and loss of life.

On the corn ration the reproduction cycle was complete and normal offspring were born.

At the time these observations on the wheat plant were made, we had no knowledge that would offer a satisfactory explanation of the results. The results showed the inadequacy of the prevailing theory of animal nutrition, but at that time added no new group of factors for completion of the theory. We did not know whether the results were to be lodged against the grain or stem portion of the plant. As our work progressed (2), it was shown that when the *yellow* corn grain was substituted for the wheat grain in our wheat grain-wheat gluten-wheat straw ration, reproduction failure continued to occur. When, however, the yellow corn-wheat gluten-wheat straw ration was fortified with a salt mixture, in which calcium was present, reproduction was normal. On the other hand, when the wheat grain-wheat gluten-wheat straw ration was supplemented with a salt mixture in which calcium was present, normal nutrition was again interfered with and there was a disturbance of the reproduction cycle. We did not at that time know that yellow corn was liberally supplied with vitamin A, while the wheat grain was but sparingly provided with this nutritive factor. These observations led us to formulate the theory that the wheat plant ration was ineffective for complete

nutrition due to an inadequate mineral content and to the presence of a toxic factor in the wheat grain.

In many experiments we had seen the serious effects of the wheat plant ration corrected by the substitution of corn stover, alfalfa, or clover hay for all or a part of the wheat straw, and the effects of a toxicity overcome. Later work in this laboratory with small animals and particularly with the wheat embryo and its oils (3) seemed to indicate the presence of a toxic substance in the oil fraction of the embryo. With a ration containing 33 per cent of ether-unextracted wheat embryo and properly supplemented with dextrin, salts, and 5 per cent of butter fat, sustained growth of rats was not obtained.

The idea that there was a toxic substance in the wheat kernel did not go unchallenged and rightly too, in view of the extensive use of this cereal in human nutrition. In 1919, Osborne and Mendel (4) published their results of an extended investigation of the nutritive properties of the wheat grain. They found that rats fed 1 year from the time of weaning on a diet containing 92 per cent of wheat or 50 per cent of commercial wheat embryo reached full maturity without giving any evidence that wheat contained a toxic substance. In reproduction, however, they were not so successful, which led them to say that "failure of rats fed 92 per cent of whole wheat to produce young of normal size is not at variance with the observations of the Wisconsin investigators." Osborne and Mendel also repeated the work with the wheat embryo and secured results at variance with those obtained in our laboratory by McCollum, Simmonds, and Pitz (3). Their rats grew normally and bore several litters of young. However, there was a relatively high mortality among the young. Their ration contained 50 per cent of wheat embryo, fortified with a suitable salt mixture, starch, and lard, but carried 22 per cent of butter fat. The differences in the results obtained in the two laboratories may rest upon a difference in the amounts of *butter fat* fed and involve the question of deficiency rather than a toxicity.

At the Ohio experiment station (5) it was also observed that the mortality of white Leghorn pullets receiving a ration containing 81.5 per cent of corn (kind not stated) was 8 per cent, while the mortality on a ration containing 81.5 per cent of wheat was 52 per cent.

In the light of our modern views of nutrition, it is perfectly clear that the wheat ration was deficient in vitamin A and in calcium. It is also strongly suggested by the response of the animals to cod liver oil, even though they were exposed to sunlight to a certain extent, that the ration was deficient in the antirachitic factor. The phosphorus content of the ration may or may not have been deficient and possibly the ration may not have carried enough iodine, although we never experienced a goitrous condition in cow or calf. Though the ration itself did not carry enough sodium and chlorine, this deficiency was taken care of by supplemental feeding with sodium chloride.

The deficiency in vitamin A in the wheat plant ration would explain the occasional blindness that was observed; its liberal presence in yellow corn (6), as contrasted with the wheat kernel, would explain the improved nutrition observed when the corn grain was substituted for the wheat grain, especially in the presence of a salt mixture containing calcium; the deficiency of calcium, although some sunlight was available, would explain the tetanies and disturbed reproductions that were seen. In fact, the question was raised whether or not the serious disturbances observed in the use of the wheat plant ration can now be explained as entirely due to deficiencies and not to inherent toxicity.

With this in mind, we have rebalanced the wheat plant ration and corrected its deficiencies as they are at present understood. The results have been as expected and are indeed very instructive. On a ration made of 8 pounds of wheat meal, 0.3 pound of wheat gluten, and 5.7 pounds of wheat straw (the original ration used), repeated failures in growth and reproduction in heifers will result. Common salt always allowed *ad libitum* will not alter these results. To balance the ration, we have added 2 per cent of bone meal (calcium phosphate); 2 per cent of raw cod liver oil; and, in these new experiments, 1 per cent of common salt has been mixed directly in the ration.

The calcium oxide content of the wheat straw used in our earlier experiments was 0.45 per cent. The wheat straw used in these experiments contained 0.36 per cent. This figure makes the calcium content of our present ration, when non-supplemented, less than that of the earlier ration used. We did not think it necessary to repeat the observations with the unsupplemented

wheat plant ration as so many records in past years have always led to the same result; namely, failure in growth and reproduction. In these new experiments, we have not repeated the use of the supplemented wheat ration during the period of growth of the



FIG. 1. No. 570. The results with the balanced ration of 1907. Protein and energy, the sole factors emphasized at that time, were supplied by the use of a ration made from wheat straw, wheat meal, and wheat gluten. Common salt was allowed *ad libitum*. The cow was shaggy coated, slow and sleepy in movement, and had a tendency to drag her hind feet. The calf was born prematurely and died. In other cases the calves were born prematurely, were extremely weak, and died soon after birth.

animal, except in one case. Instead, we have used mature animals, involving them in reproduction and milk production, which are quite as crucial tests of the nutritive completeness of a ration as is growth, and possibly are even more crucial.

Records of Individual Cows.

No. 656, a grade Holstein, had been in our experimental herd since 1916 under observation on the influence of various natural rations on reproduction and milk production. During this time she had produced seven calves of varying degrees of weight and vigor. On June 29, 1923, she was placed on our wheat ration fortified with bone meal, common salt, and raw cod liver oil as previously described. We had no trouble in securing consumption of the ration although some individuals may refuse to eat it due

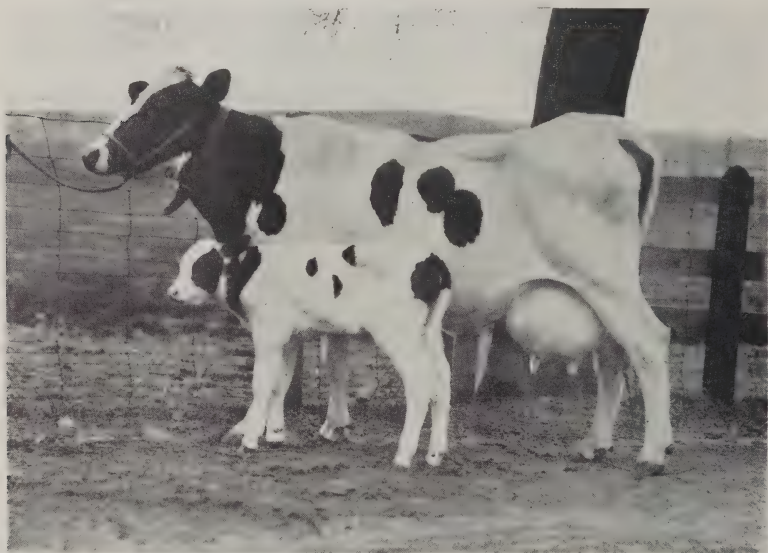


FIG. 2. No. 656. The result with the balanced ration of 1924; the same wheat ration proportions as used in 1907, but supplemented with bone meal 2 per cent, common salt 1 per cent, and raw cod liver oil 2 per cent. The cow was sleek in appearance, active, and apparently in normal nutritive condition. A normal strong calf (115 pounds at birth) was the result from this ration.

to the taste of the cod liver oil. The supplementing materials were all mixed intimately in the grain portion of the ration and a new mixture was prepared every 2 weeks. This animal, as in the treatment of the entire herd, had access to an outdoor, vegetation-free, paddock whenever the weather would permit. Consequently there was a variable amount of exposure to sunlight as in our preceding experiments.

The animal was milked until Dec. 24, 1923, when she was dried off. She was due to freshen Apr. 7, 1924, and did freshen Apr. 16, 1924, giving birth

to a male calf of 115 pounds weight. The calf was strong and vigorous and in marked contrast to what would have been the result had the supplementing materials been omitted from the ration. Further, the calf was not prematurely born as would have been the case on a non-supplemented wheat ration, but was carried the full term. The cow cleaned naturally which is a further contrast to what generally occurred in the previous experiments. Fig. 1 shows a reproduction failure on the original wheat ration of 1907 and Fig. 2 the result secured with the supplemented wheat ration in the case of this cow.



FIG. 3. No. 693. Another result with the wheat ration supplemented with bone meal 2 per cent, common salt 1 per cent, and raw cod liver oil 2 per cent. Strong, healthy calves (twins) were produced. The cow was in excellent nutritive condition. The twins weighed 124 pounds.

The calf was allowed to suckle the mother and when 4 weeks old weighed 196 pounds or had gained in weight at a rate of 3 pounds per day, which is well above the average weight even for Holstein calves. During the gestation period the cow had remained in excellent physical condition with a loose, pliable, sleek coat and with every indication of complete nutrition. She was free from any infection such as *Bacillus abortus* or tuberculosis. After the calf was removed from the cow, milk records were kept and this individual continued for several months to produce 30 to 40 pounds of milk daily. Heavy milk production was not expected as the protein supply was only of moderate quality and quantity.

No. 693. This grade Holstein had been in our experimental herd since January, 1922, and had produced two calves of varying degrees of vigor

on restricted natural rations. In December, 1923, she was placed on the wheat ration, fortified with bone meal, common salt, and raw cod liver oil, the same ration as fed No. 656. She ate the ration without trouble,—the cod liver oil being intimately mixed with the grain. This animal remained in good thrifty condition and, on Dec. 10, 1923, was bred. She was milked until Aug. 24, when she became dry. On Sept. 8, 1924, she gave birth to twin calves (a heifer 58 pounds and a bull 66 pounds),—both strong and active. These calves suckled the mother within a half hour after birth. The mother had remained in splendid nutritive condition during the entire gestation period and cleaned naturally. This is another example of the marked contrast in the effect of the wheat ration, supplemented and non-supplemented, on the nutritive status and reproducing capacity of this species. (See Fig. 3.)

CONCLUSIONS.

It is apparent from our data accumulated with fuller knowledge of the factors of nutrition that the wheat plant ration can be made nutritively complete by specific additions which make good its deficiencies. This being the case it does not seem necessary to assume the presence of an inherent toxic factor in the wheat grain in explanation of the nutritive failure following the use of a ration made wholly from the wheat plant.

To assume that the organism can overcome an inherent toxicity of the wheat grain when the wheat plant ration is fortified with its deficiencies is, we believe, a wholly unnecessary assumption, although, of course, it is a remote possibility.

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ESTIMATION OF THE SURFACE AREA OF THE FETUS, AND A GRAPHIC COMPARISON OF THE VARIOUS SURFACE AREA FORMULAS.

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Estimation of the Surface Area of the Fetus.

If the rate of heat production is proportional to the active protoplasmic mass, and if the surface area offers the most practical method available for estimating the active protoplasmic mass, as maintained by Boothby and Sandiford (1922, 1924), then it is obviously necessary, when estimating the heat production of a pregnant woman, to take into consideration the surface area of the fetus as well as that of the mother. Therefore, in the case of the pregnant woman, in order to derive the true basis for calories for each square meter each hour, the total calories each hour must be divided by the sum of the surface area of the fetus and of the mother, the latter being calculated on the basis of the weight of the mother minus the weight of the fetus. The average weight of the fetus for each lunar month, and the normal maximal and minimal variations, have been determined by Streeter. The figures given in the "Text-book of embryology" by Prentiss and Arey are quoted from Jackson for weight, and from Mall for crown-heel length. The data for the weight of the fetus, when plotted on logarithmic paper, lie on a straight line, as can be seen in Chart 1, Curve B. Therefore, at any period of pregnancy, the weight of the fetus can be estimated with considerable accuracy, especially if the weight at birth is known. If the weight at birth is distinctly greater or less than that shown as the average weight, a line may be constructed passing through the point corresponding to the weight at birth and parallel to the average line. In most instances, however, the use of the average line will

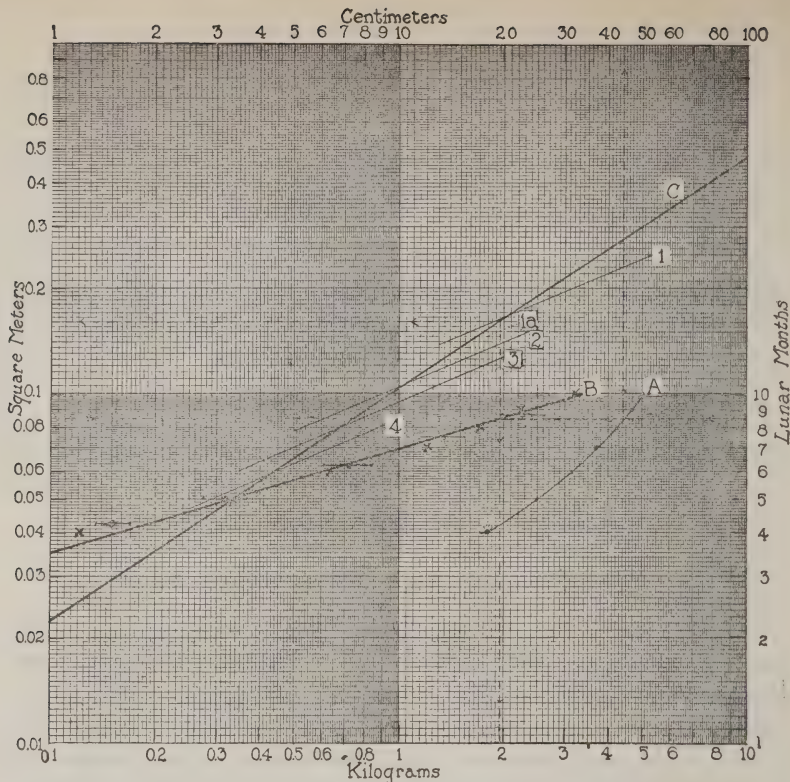


CHART 1. *Weight, length, and surface area of the fetus for the various lunar months.* The solid circles in Curve A represent the crown-heel length of the fetus in centimeters (upper abscissa) as quoted from Mall by Prentiss and Arey.¹ The crosses on Curve B are the weights in kilos of the fetus as quoted from Jackson by Prentiss and Arey,¹ and the large solid circles represent the average weights as given by Streeter with the maximal and minimal weights for the age indicated by the line ending in small solid circles. The length on the upper abscissa in centimeters and the weight on the lower abscissa in kilos are plotted against the right ordinates in months. Curve C is the surface area of the fetus calculated by the Lissauer formula ($S.A. = 10.3 \times \sqrt[3]{W^2}$) with the left ordinate representing surface area and the lower abscissa, kilos. Curves 1, 2, 3, and 4 represent the surface area calculated by the Du Bois formula ($S.A. = W^{0.425} \times H^{0.725} \times 71.84$) for lengths of 50, 40, 35, and 30 cm. To use the chart, locate the calculated age of the fetus on the right ordinate, for example $8\frac{1}{2}$ lunar months, and read off on Curve B the corresponding weight on the lower abscissa, for example 1.95 kilos. From the weight erect a perpendicular to the surface area line, Curve C, from which obtain on the left ordinate the surface area, 0.160. Both length and weight can be taken into consideration and the Du Bois formula used. In the example given above, the corresponding length would be 45 cm. and the surface area interpolated between Curves 1 and 2, as shown by the short dotted line, Curve 1a, gives the surface area of 0.150, a negligible variation from the Lissauer value.

The lower abscissa for the quadrants runs from 0.1 to 10 kilos and the upper abscissa from 1 to 100 cm. The left ordinate runs from 0.01 to 1 sq. m., and the right ordinate from 1 to 10 lunar months.

¹ Prentiss and Arey (1922), p. 90.

be sufficient. If the total length of the fetus, as given by Mall, for each lunar month is plotted, Curve *A* is obtained; this, unlike the weight curve, is not a straight line.

The surface area of infants can be determined with considerable accuracy by the Lissauer formula. This is supported by the fact that the Du Bois formula gives for the significant ranges similar results for a given height. It seems legitimate, therefore, to extend the Lissauer formula to include the surface area of the fetus for any given weight, and the points have been plotted as Curve *C* of Chart 1. In Curves 1, 2, 3, and 4 have been plotted the surface areas derived by the Du Bois formula for the heights 50, 40, 35, and 30 cm., respectively, to show the similarity of the results obtained by the two formulas.

In using the chart, the calculated age of the fetus is located on the right ordinate of Curve *B*, and the corresponding weight obtained on the lower abscissa. From the weight, a perpendicular is erected to Curve *C*, and the surface area read off on the left ordinate. If desired, both the weight and length of the fetus can be taken into consideration, and the surface area estimated from the Du Bois curves given in this chart. It is thus possible to estimate at a glance the surface area of the fetus throughout the course of pregnancy with a fair degree of accuracy. Besides the intrinsic error in all surface area formulas, the usual custom of neglecting the surface area of the fetus introduces a special and consistent error in predicting the combined surface area of mother and fetus of 10 to 15 per cent. This error is reduced to at least one-third this value by estimating the surface area of the fetus according to the method suggested here in spite of the fact that there may be a considerable error (as large as 20 per cent) in estimating the weight and surface area of the fetus. The application of this calculation will be described in the following paper by Sandiford and Wheeler.

Graphic Comparison of the Various Surface Area Formulas.

A graphic representation is a material help in the understanding of the fundamental relationship of, as well as the detailed differences between, the various surface area formulas. Therefore, in Chart 2 are shown the position and the type of curves obtained by plotting on logarithmic paper the surface areas calculated by

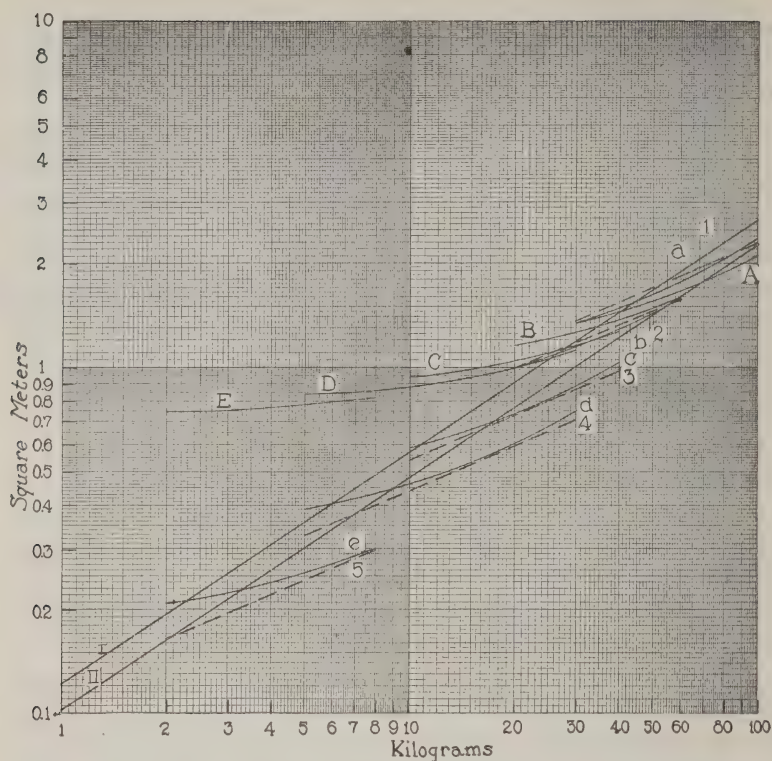


CHART 2. *Surface areas derived by the various surface area formulas*
 Curves I and II represent the surface areas derived from the Meeh ($S.A. = 12.3 \times \sqrt[3]{W^2}$) and the Lissauer ($S.A. = 10.3 \times \sqrt[3]{W^2}$) formulas, respectively. The broken lines, 1, 2, 3, 4, and 5 are the surface areas calculated by the Du Bois formula ($S.A. = W^{0.425} \times H^{0.725} \times 71.84$) for heights of 190, 150, 100, 75, and 50 cm., respectively. The solid curved lines *a*, *b*, *c*, *d*, and *e* represent the surface areas derived by rearrangement of the Harris and Benedict heat prediction formula for males made by Boothby and Sandiford ($S.A. = \frac{2.75W + S - 15.1}{190}$) for the heights 190, 150, 100, 75, and 50 cm., respectively. The solid curved lines *A*, *B*, *C*, *D*, and *E* represent the surface areas derived by rearrangement of the Harris and Benedict heat prediction formula for females, made by Boothby and Sandiford

$$\left(S.A. = \frac{5.17W + S + 301}{480} \right)$$

for the heights 190, 150, 100, 75, and 50 cm., respectively.

The abscissa for the quadrants runs from 1 to 100 kilos and the ordinates from 0.1 to 10 sq. m.

means of the various surface area formulas, including those derived by the rearrangement made by Boothby and Sandiford of the Harris and Benedict heat prediction formulas. The long solid lines, Curves I and II, representing the Meeh and Lissauer formulas, respectively, cross the broken lines for the Du Bois formula, representing the surface area for different heights (Curves 1, 2, 3, 4, and 5) in the region of the average weight for a given height, thus bringing out the general applicability of the simple formula based on the two-thirds power of the weight. However, the less steep slope of the Du Bois curves shows why comparison of the surface area between individuals of different weights, but of the same height, is more accurately determined by the Du Bois formula which takes into consideration the effect of height. The curves representing the Harris and Benedict formulas, rearranged to solve the surface area, likewise are in close relationship to those representing the Du Bois, Meeh, and Lissauer formulas within the limits of height and weight studied by Harris and Benedict (Curves *a* and *b* for men, and Curves *A* and *B* for women). The position of the Harris and Benedict lines, Curves *C*, *D*, and *E*, representing the surface area of females of 100, 75, and 50 cm. in height, illustrates why these authors do not recommend the extension to small individuals of the heat prediction formula for females, and suggests that there may also be a greater error in the lower part of Curve *B* than in Curve *b* or 2. The Harris and Benedict curves for males of 100, 75, and 50 cm. (Curves *c*, *d*, and *e*) are, however, very close to the corresponding Du Bois curves.

We agree with Krogh that these formulas represent functions of weight alone or of height and weight combined, and it is a matter of indifference for the mathematician that they predict the surface area while at the same time they form the best basis yet obtained for estimating the heat production of normal persons. However, as pointed out by Boothby and Sandiford, the underlying basis for these correlations seems to be first, that the heat production of a person under standard and normal physiologic conditions varies according to the mass of active protoplasmic tissue, and second, that this mass of active protoplasmic tissue is dependent on certain functions of the height and weight, but only if the value of these functions is such that they correspond to the

surface area of the organism. Unfortunately, in the practical application to metabolism the surface area must be deduced from the easily determined factors of weight alone, or better, from height and weight, although if the surface area were a readily measurable factor, we could with equal propriety and possibly with even better results use this measurement directly for estimating the relative mass of active protoplasmic tissue. Consequently it seems not only permissible but also correct for the biologist to retain the long established and convenient custom of expressing the heat production on the basis of calories for each square meter for comparison with the standards for age and sex.

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THE BASAL METABOLISM BEFORE, DURING, AND AFTER PREGNANCY.

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Several observers have reported that the total energy production of the normal pregnant woman undergoes little or no change in the beginning of gestation, but that during the latter part of pregnancy, there is a gradual and significant increase. We have studied the heat production in one woman before conception, during pregnancy and the puerperium, and for 4 months after the cessation of lactation and the reestablishment of menstruation, a total period of 17 months. Our observations, as will be pointed out in detail, confirm the finding of a definite increase in the total heat production during the latter part of pregnancy, but we believe that our data, as well as those of other observers, show that the rate of metabolism of a unit mass of the mother's tissue undergoes no material change, and that the increase in the total heat production may be accounted for by the increase in the amount of active protoplasmic tissue, which is composed chiefly of the fetus, with a small amount of new and accessory tissue of the mother.

The subject of this study was a normal multipara (para IV), aged 34 years. The last menstrual period before pregnancy occurred March 3, 1923, and parturition took place December 7, making a period of gestation of approximately 10 months' duration. The pregnancy was normal, clinically, and examinations of the urine were negative. The blood pressure readings taken at the time of each metabolism test were low normals. Three or four times during the 5th and 6th months there was slight nausea in the afternoon. This symptom had been present in diminishing degree in each of the four pregnancies. Vomiting occurred

but once, an afternoon in the 5th month (July 10, 1923) after the patient had eaten raspberries on an empty stomach. From the beginning of the 5th through the 7th lunar month, there were occasional days of lassitude, when the subject felt drowsy and became exhausted on the slightest exertion. Except for this rather indefinite disability, pressure pains, and some edema of the ankles during the last month, the subject was in very good health during the pregnancy. Confinement occurred December 7, 1923. The baby girl weighed at birth 3.6 kilos, the weight of the fluid was 0.9 kilo, and of the blood and placenta 1.8 kilos. Lactation was of approximately 4 months' duration. Five feedings were given every 24 hours, and the amount of milk produced increased gradually from 174 gm. at 3 days postpartum to 623 gm. on the 23rd day. The supply, however, was not adequate for the baby, and complementary feedings were begun at the end of the 1st week. In the 4th week of the puerperium, the mother contracted an infection of the upper respiratory tract which was severe for about 5 days (January 5 to 10, 1924) and did not entirely clear up for 2 weeks. During this time the average daily amount of milk was only 439 gm. During the 6th week there was a daily average of 504 gm., then the amount gradually diminished. Because the baby persistently refused to nurse, she was weaned in the 16th week. The mother led an active, normal life, and no attempt was made to regulate the diet or the amount of exercise.

The metabolism was determined with the subject under standard postabsorptive conditions after complete rest in bed for $\frac{1}{2}$ hour. As the subject was calm and quite undisturbed by the numerous metabolism tests, and cooperated fully at all times, the results are satisfactory from this standpoint. For the determination of the basal metabolic rates, the expired air was collected in a gasometer by means of a mask and valves, and analyzed in duplicate in the Haldane gas analysis apparatus. All readings were taken in duplicate by two observers, and the calculations checked by two persons. The details of the technique need not be reviewed here since they have been fully described by Boothby and Sandiford.

Complete data on this study are given in Tables I and II. In Chart 1 are plotted the total calories for each hour (Curve *D*),

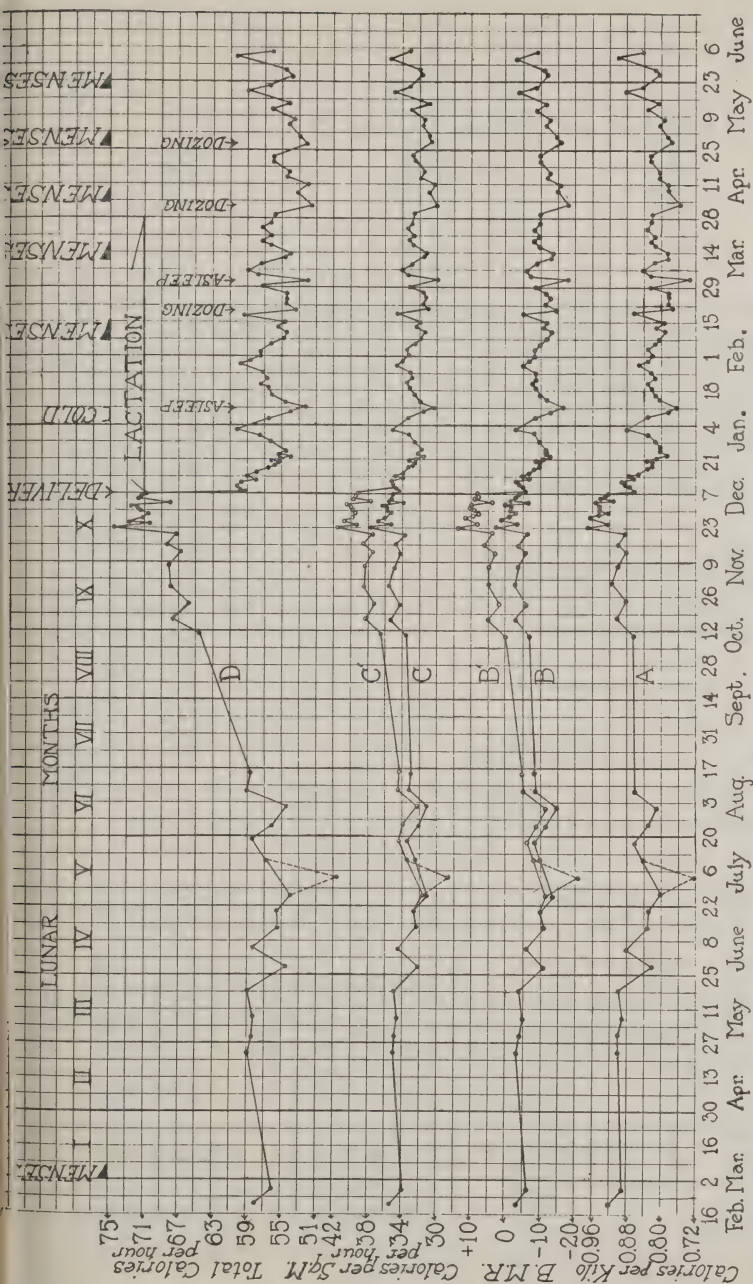


CHART 1. Basal metabolism findings before, during, and after pregnancy. The subject, a woman, aged 34 years, height 160 cm. Curve A represents the calories for each kilo; Curve B, the basal metabolic rate calculated during the course of pregnancy, by dividing the total calories each hour by the sum of the surface area of the mother and fetus, and comparing the result obtained with the Du Bois normal of the mother, 36.5 calories. Curve B' represents the basal metabolic rate calculated in the usual method, using the Du Bois surface area and normal standards. Curve C is the calories for each square meter each hour derived for the course of pregnancy as just described for Curve B. Curve C' represents the calories for each square meter each hour, obtained by dividing the total calories each hour by the Du Bois surface area obtained by using the total weight of mother and fetus in the usual manner. Curve D represents the total calories for each hour.

TABLE I.
Basal Metabolism Findings before and during Pregnancy.
Subject: A woman, aged 34 years, height 160 cm.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Remarks.
Date.	Weight. kg.	Temperature. °F.	Pulse.	Respiratory quotient.	Total calories each hr.	Calories for each sq. m. each hr. (Du Bois).	Basal metabolic rate (Du Bois). per cent	Estimated surface area of fetus (Lissauer). sq. m.	Estimated weight of fetus. kg.	Estimated weight of mother (Column 2 - Column 10). kg.	Estimated surface area of mother alone (Du Bois) (from Column 11). sq. m.	Surface area of mother and fetus (Column 9 + Column 12). sq. m.	Calories for each sq. m., using surface area of mother and child (Column 6 + Column 13). sq. m.	Basal metabolic rate of Column 14, using normal of mother (Du Bois). per cent	
1923															
Feb. 21	62.9	98.0	70	0.80	57.9	35.3	-3							-3*	
" 27	62.7	98.6	63	0.88	55.7	34.0	-7							-7*	
Apr. 23	65.7	98.5	66	0.82	58.9	35.1	-3							-3*	
" 30	65.2	98.6	67	0.81	58.5	35.0	-4							-4*	
May 7	65.5	98.4	73	0.80	58.3	34.7	-5							-5*	
" 18	65.6	98.4	71	0.81	59.0	35.1	-4							-4*	
" 28	66.1	98.4	65	0.82	54.5	32.3	-11							-11*	
June 4	66.5	98.5	70	0.80	58.4	34.5	-6							-6*	
" 13	66.9	98.3	66	0.82	55.3	32.5	-11							-11*	
" 20	67.1	98.4	71	0.83	55.5	32.7	-10							-10*	
" 27	67.2	98.2	70	0.83	54.0	31.8	-12	0.04	0.2	67.0	1.69	1.73	31.2	-14	
July 4	67.7	98.0	73	0.80	48.7?	28.6?	-21?								
" 11	67.6	98.6	65	0.80	56.9	33.5	-8	0.04	0.2	67.4	1.70	1.74	32.7	-10	Mar. 3. Beginning of menstrual period.
" 18	67.9	98.2	70	0.80	58.4	34.4	-6	0.05	0.3	67.6	1.70	1.75	33.4	-8	July 4. Leak in mask.

* Same as in Column 8, because the mass of the fetus was too small to justify recalculation.

Dec. 7. Confined at 4.00 p.m.

July 25	67.5	97.8	68	0.75	56.1	33.0	-9	0.05	0.3	67.2	1.70	1.75	32.1	-12
Aug. 1	67.0	98.1	72	0.78	54.4	32.2	-12	0.06	0.4	66.6	1.69	1.75	31.1	-15
" 8	68.5	98.4	70	0.86	59.1	34.5	-5	0.07	0.5	68.0	1.71	1.78	33.2	-9
" 15	68.5	98.6	80	0.87	58.8	34.4	-5	0.07	0.6	67.9	1.71	1.78	33.0	-9
Oct. 10	74.9	98.2	79	0.85	64.5	36.4	0	0.15	1.7	73.2	1.76	1.91	33.8	-7
" 17	74.5	97.9	83	0.94	67.4	38.1	+5	0.16	1.9	72.6	1.75	1.91	35.3	-3
" 24	74.3	97.4	85	0.87	65.6	37.1	+2	0.16	1.9	72.4	1.75	1.91	34.3	-6
" 31	74.9	98.1	85	0.86	67.9	38.3	+5	0.17	2.1	72.8	1.75	1.92	35.4	-3
Nov. 7	75.3	98.0	83	0.82	68.0	38.2	+5	0.20	2.7	72.6	1.75	1.95	34.9	-4
" 13	75.8	96.8	90	0.88	66.8	37.5	+3	0.20	2.7	73.1	1.76	1.96	34.1	-6
" 16	75.8	96.8	81	0.86	68.4	38.4	+6	0.22	3.0	72.8	1.75	1.97	34.7	-4
" 20	76.2	98.2	83	0.83	67.2	37.5	+4	0.22	3.0	73.2	1.76	1.98	33.9	-7
" 23	76.3	98.0	83	0.84	74.3	41.5	+14	0.23	3.3	73.0	1.75	1.98	37.5	+3
" 24	76.5	98.0	93	0.88	70.1	39.2	+8	0.23	3.3	73.2	1.76	1.99	35.2	-4
" 27	76.1	97.9	91	0.77	72.1	40.3	+11	0.23	3.3	72.8	1.75	1.98	35.4	-3
" 28	76.0	96.8	92	0.84	69.8	40.8	+13	0.23	3.3	72.7	1.75	1.98	36.9	+1
" 29	76.5	97.6	81	0.86	72.4	40.5	+11	0.25	3.6	72.9	1.75	2.00	36.6	+1
" 30	76.3	98.2	82	0.83	71.1	39.7	+9	0.25	3.6	72.7	1.75	2.00	34.8	-4
Dec. 1	76.3	98.2	92	0.85	72.8	40.4	+11	0.25	3.6	72.7	1.75	2.00	35.6	-2
" 3	76.3	97.6	92	0.87	70.2	39.0	+7	0.25	3.6	72.7	1.75	2.00	35.1	-4
" 4	76.2	97.8	90	0.82	73.4	40.8	+12	0.25	3.6	72.7	1.75	2.00	36.7	+1
" 5	76.2	97.8	74	0.86	72.6	40.3	+10	0.25	3.6	72.7	1.75	2.00	36.3	0
" 6	76.2	97.8	90	0.88	67.9	37.7	+4	0.25	3.6	72.6	1.75	2.00	34.0	-7
" 7	76.2	97.8	80	0.86	71.5	39.7	+9	0.26	3.9	72.3	1.75	2.01	35.6	-2
" 8	76.9	97.8	80	0.85	71.2	39.5	+8	0.26	3.9	73.0	1.75	2.01	35.4	-3
" 9	77.1	98.1	83	0.84	70.9	39.4	+8	0.26	3.9	73.2	1.76	2.02	35.1	-4

TABLE II.
Basal Metabolism Findings during 6 Months Following Delivery.

Date.	Weight.	Temperature.	Pulse.	Respiratory quotient.	Total calories for each hr.	Calories each sq.m. each hr. (Du Bois).	Basal metabolic rate (Du Bois).	Remarks.
1923	kg.	°F.					per cent	
Dec. 8		97.8	67	0.80	59.2	34.4	-6	Dec. 7, Delivery of baby weighing 3.6 kg.
" 10	69.2	97.8	74	0.84	60.2	35.0	-4	
			73	0.83	59.5	34.6	-5	
" 11	68.4	98.0	80	0.82	60.5	35.4	-3	
			80	0.82	59.8	35.0	-4	
" 12	67.5	97.6	78	0.86	59.7	35.2	-3	
			77	0.85	59.9	35.3	-3	
" 13	67.3	97.8	78	0.91	57.2	33.7	-7	
			77	0.89	58.4	34.3	-6	
" 14	67.8	97.8	74	0.94	59.0	34.7	-5	
" 15	67.8	98.1	74	0.92	57.8	34.0	-7	
" 17	68.0	97.8	63	0.90	56.6	33.3	-8	
" 18	67.9	97.8	65	0.89	55.6	32.7	-10	
" 19	67.9	97.8	63	0.89	55.4	32.6	-10	
" 20	68.0	97.9	64	0.89	56.1	33.0	-9	
" 21	68.0	98.0	63	0.90	55.1	32.4	-11	
" 22	68.6	98.0	59	0.87	53.8	31.6	-13	
" 24	68.9	98.0	58	0.92	55.2	32.3	-12	
" 26	68.6	97.4	55	0.85	54.6	31.9	-12	
" 28	69.1	98.0	60	0.84	56.2	32.7	-10	
" 31	69.4	98.6	66	0.88	57.4	33.4	-8	
1924								
Jan. 2	69.0	99.2	76	0.79	60.4	35.3	-3	Jan. 2. Pharyngitis.
" 7	68.6	98.1	69	0.83	56.7	33.1	-9	" 7. "
" 9	68.9	97.7	64	0.84	53.9	31.5	-13	" 9. "
" 11	69.0	97.0	56	0.79	52.1	30.3	-17	" 11. Asleep.
" 14	68.6	97.4	67	0.81	54.7	32.0	-12	
" 16	69.1	97.8	65	0.83	56.2	32.7	-10	
" 19	68.8	97.3	70	0.82	56.7	33.1	-9	
" 21	69.1	97.4	59	0.87	57.5	33.5	-8	
" 23	69.9	98.0	61	0.85	56.8	33.0	-9	
" 25	69.6	97.4	62	0.85	57.1	33.2	-9	
" 28	70.1	97.2	62	0.86	59.7	34.7	-5	
" 30	70.4	97.2	65	0.85	58.7	34.1	-7	
Feb. 1	70.4	97.5	64	0.83	57.5	33.4	-8	
" 4	68.9	97.8	59	0.80	57.4	33.6	-8	
" 6	69.5	97.6	57	0.80	56.2	32.7	-10	Feb. 6. Beginning of menstrual period.
" 8	68.8	97.2	56	0.86	54.8	32.0	-12	

TABLE II—*Concluded.*

Date.		Weight.	Temperature.	Pulse.	Respiratory quotient.	Total calories for each hr.	Calories each sq.m. each hr. (Du Bois).	Basal metabolic rate (Du Bois).	Remarks.
1924		kg.	°F.					per cent	
Feb. 11		69.1	97.4	57	0.85	54.5	31.7	—13	
" 13		68.9	98.0	58	0.84	55.5	32.5	—11	
" 15		68.9	96.0	56	0.83	54.7	32.0	—12	
" 18		69.1	97.4	65	0.81	59.2	34.6	—5	
" 20		69.4	98.0	54	0.82	53.1	31.1	—15	Dozing.
" 22		69.6	97.6	57	0.83	54.6	31.8	—12	
" 25		69.5	97.4	58	0.86	54.4	31.6	—13	
" 27		69.7	97.5	62	0.80	54.4	31.8	—12	
" 29		70.0	97.2	60	0.85	57.1	33.2	—9	
Mar. 3		70.7	97.3	53	0.82	51.9	30.0	—18	Asleep.
" 5		70.2	97.3	64	0.80	57.9	33.7	—7	
" 7		70.2	96.9	63	0.83	59.0	34.1	—6	
" 10		70.4	98.6	68	0.83	57.3	33.1	—9	Mar. 11. Beginning of menstrual period.
" 12		70.4	97.6	59	0.84	54.6	31.6	—13	
" 14		69.5	97.4	62	0.82	54.0	31.4	—14	
" 17		69.3	97.8	59	0.81	56.4	32.8	—10	
" 19		69.6	97.7	64	0.73	57.2	33.3	—8	
" 21		69.1	97.6	63	0.78	56.1	32.6	—10	
" 24		68.5	97.8	72	0.72	57.1	33.4	—8	
" 27		68.8	97.7	60	0.79	56.3	32.9	—10	
" 31		67.8	98.2	61	0.79	55.6	32.7	—10	
Apr. 3		68.8	98.6	62	0.84	51.3	30.0	—18	Apr. 4. Beginning of menstrual period.
" 9		68.2	97.8	54	0.79	53.1	31.0	—15	
" 11		68.2	98.0	60	0.80	51.9	30.4	—16	Apr. 11. Very drowsy.
" 14		67.8	97.7	61	0.81	54.4	32.0	—12	
" 16		68.1	97.8	64	0.78	54.3	31.7	—13	
" 21		68.2	98.2	58	0.78	55.9	32.7	—10	
" 23		67.9	98.5	61	0.80	55.8	32.9	—10	
" 28		67.7	97.4	59	0.83	52.1	30.6	—16	Apr. 27. Beginning of menstrual period. Drowsy.
" 30		68.0	97.9	60	0.84	52.9	30.9	—15	
May 5		67.5	97.8	60	0.77	54.1	31.8	—12	May 5. Very tired.
" 7		68.0	97.6	63	0.81	53.4	31.6	—13	
" 12		67.2	97.9	61	0.79	56.1	33.0	—9	
" 14		67.6	98.4	64	0.82	54.1	31.8	—12	
" 19		67.2	97.8	64	0.82	59.0	34.9	—4	May 20. Beginning of menstrual period.
" 21		67.5	97.6	60	0.81	56.4	33.2	—9	
" 26		66.8	97.8	59	0.78	53.7	31.8	—12	
" 28		67.5	97.6	65	0.81	54.5	32.1	—11	
June 2		67.2	98.0	61	0.75	60.2	35.4	—3	June 2. Restless night.
" 4		67.1	98.2	59	0.79	56.2	33.1	—9	

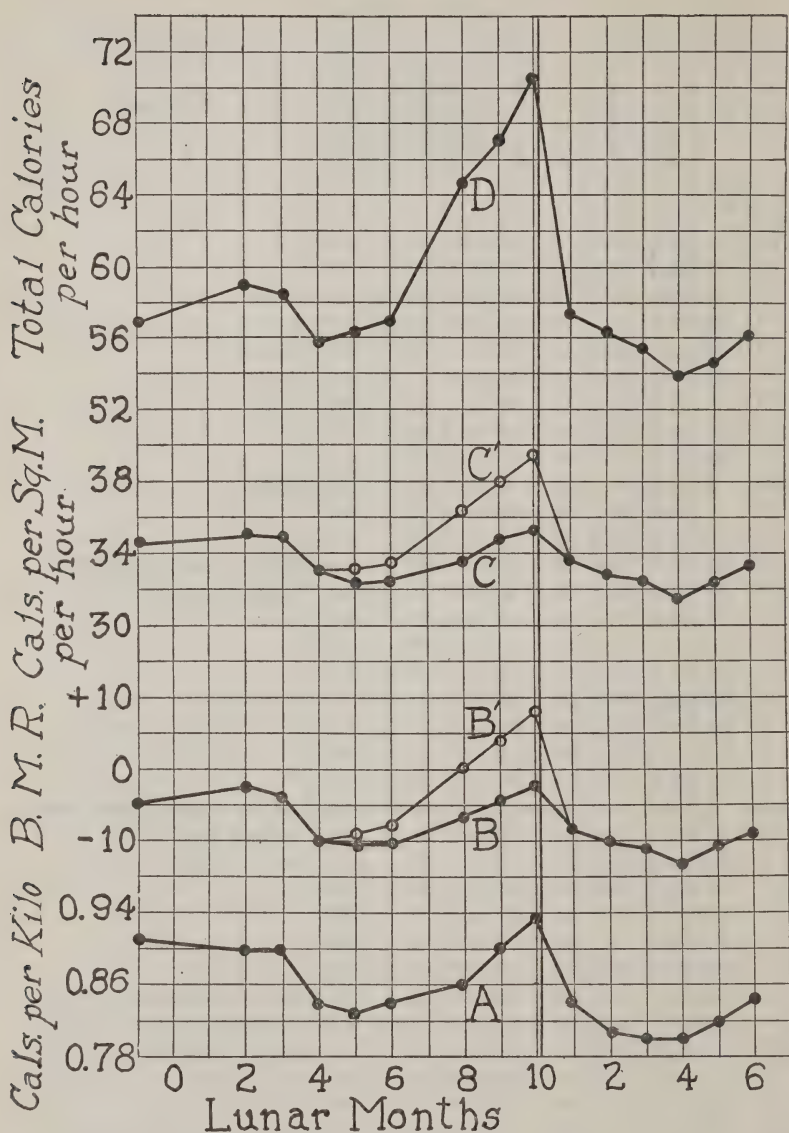


CHART 2. Basal metabolism findings averaged according to lunar months before, during, and after pregnancy. The subject, a woman, aged 34 years, height 160 cm. Curves A, B, B', C, C', and D represent the averages for each lunar month of the corresponding Curves A, B, B', C, C', and D in Chart 1.

the calories for each square meter (Curve *C'*) and each kilo (Curve *A*) each hour, and the basal metabolic rate (Curve *B'*), calculated in the ordinary manner, using the Du Bois standards. In addition are plotted the two curves, *C* and *B*, representing calories for each square meter each hour, and basal metabolic rate, respectively, calculated according to the new method described in this study. The averages for each lunar month of each of these curves, before and after pregnancy, are plotted in Chart 2. The only observation omitted from the average was that of July 4, which was so far below the level of any other determination that an experimental error seemed probable, although on that date the subject was very weak and nearly fainted. The metabolism studies were begun during the latter part of February, 1923, and two control periods previous to conception were obtained. On account of the subject's absence from town, no determinations were made during the 1st month, and only one determination during the 2nd lunar month of pregnancy. From the 3rd to the 9th lunar month, inclusive, the metabolism was determined once a week, except during the 7th month, when the subject was again out of town. During this month, there also occurred 2 days of great lassitude (September 4 and 5). On the 4th, in particular, there was drowsiness with marked subjective disinclination for any exertion; this was the only day spent in bed during the entire pregnancy. The subject at the time was near New Haven, and through the courtesy of the Yale Metabolic Laboratory a metabolic rate was made September 6. This test was made in duplicate, with results of -19 and -18 per cent. Rates taken at the same laboratory 9 and 25 days later were -12 and -2 per cent, respectively, each being the average of two observations varying within 5 per cent of each other. In the beginning of the 10th month, three determinations were made each week, and from November 24 to December 3 the rates were taken daily (except Sunday) in duplicate, and from December 3 until confinement, December 7, single daily determinations were made. From December 8 to 14, duplicate determinations were made daily, then single determinations daily until December 24, when the metabolism was observed three times a week until March 24; after that two determinations were made each week until the end of the study, 6 months after delivery.

As can be seen in Chart 2, which represents the average for each lunar month, there was less than a ± 3 per cent monthly variation from the average of the total energy production during the first 6 months of pregnancy. During the 8th month there was a gradual increase in the total energy production, and the peak was reached during the last month. The height of the latter was somewhat influenced by fetal movements which in a few of the periods were quite marked, as can be seen in Chart 1 by the greater irregularity in the daily determinations of the last month of pregnancy. Up to November 23, movements of the fetus during the metabolism tests had scarcely been perceptible. On that day there were energetic, and almost continuous, fetal movements during the rest period, as well as during the test itself. As Chart 1 shows, the metabolism on that day was the highest obtained. The influence of the fetal movements on the heat production is, therefore, very evident. During each subsequent test, a rough estimate of the duration and intensity of the fetal movements was made by the subject signalling to the observer by slightly bending one finger when the fetal movements were very mild, two fingers when they were more vigorous, and subsequently straightening the fingers when the fetus was again quiet. A study of these records shows that there is a slight increase in the metabolism, roughly corresponding to the intensity and duration of the fetal movements.

On the day of delivery the subject's weight had increased 23 per cent over that during the control period, and there was an increase of 25 per cent in the total heat production; hence there was practically no change in the calories for each kilo. Although there was an increase in the total heat production from 56.8 calories, during the control period, to 59.2 calories on the morning following delivery, as shown in Chart 1 and in Tables I and II, the basal metabolic rate, which takes into consideration variations in weight, on the morning following delivery was -6 per cent, corresponding to the patient's basal rate of -5 per cent before pregnancy, which is confirmatory evidence that the mother's rate of heat production for each unit mass of tissue remained unchanged during pregnancy, and that the increases in total heat production were due to the metabolism of the new tissue.

Basal metabolism determinations were made for 6 months after delivery and, as shown in Chart 1 and in Table II, the basal metabolic rates show fluctuations of the same magnitude as reported by Benedict, by Blunt and Dye, by Sturgis, and others. The fluctuations are of slight magnitude, and are not correlated with the menstrual cycle. The total heat production and basal metabolic rates, when averaged for each lunar month, show a slight decrease, so that 4 months after delivery the metabolism was approximately 8 per cent lower than before pregnancy. This may be accounted for by the less active life of the mother, necessitated by nursing, especially since the metabolism tended to return to normal during the next 2 months as her usual activities were resumed following the cessation of lactation. Our data confirm the findings of Hasselbalch and of Carpenter and Murlin that lactation in the human being is not associated with an increase in heat production, and that, therefore, the conversion of the mother's food into milk does not involve a material loss of energy. Lusk points out that the rearrangement of food materials in the preparation of milk depends on hydrolytic changes and syntheses which involve hardly any thermol reactions.

The experiments of Magnus-Levy, Zuntz, Hasselbalch, Carpenter and Murlin, Root and Root, Rowe, Alcott, and Mortimer, and our report show beyond question that the total energy production of a pregnant woman increases slightly, beginning at the middle of gestation, and finally reaching a maximum of approximately 20 per cent above her basal value before pregnancy. However, we do not believe that there is definite evidence that the rate of heat production of a unit mass of tissue of the normal human organism is materially changed during pregnancy, but rather that such increases as occur represent the heat production of the newly formed protoplasmic tissue, composed largely of the fetus, and to a less extent of maternal tissue.

Carpenter and Murlin, using the respiration calorimeter, have shown that the energy production of mother and child suffers no deflection at birth, and that, therefore, the heat production of the fetus at term is practically uninfluenced by birth (Table III). In consequence, the fetus, as well as the baby after birth, should be regarded as a being independent of the mother, and its heat production calculated on its own surface area, which is propor-

tional to its own active protoplasmic mass before and after delivery. We have made a series of such calculations which are summarized in Table I. If the weight values of the fetus at different months of pregnancy are plotted on logarithmic paper, they are found to lie on a straight line, as shown by Sandiford. Therefore, it is possible to read, with a fair degree of accuracy, the probable weight of the fetus at any period of pregnancy, and the surface area of the fetus can then be readily calculated by Lissauer's formula, or read directly from Chart 1 in the preceding paper by Sandiford. The weight of the mother alone during the course of pregnancy can be obtained, therefore, by subtracting the estimated weight of the fetus from that of the mother and fetus. From this estimated weight of the mother,

TABLE III.

Heat Production of Mother and Baby before and after Delivery.

Data of Murlin and Carpenter using the respiration calorimeter.

Case No.	1	2	3	4
	Average total calories of mother and child each hr. after delivery.	Average total calories of mother each hr. after delivery.	Average total calories of mother and fetus each hr. just before delivery.	Percentage variation of Column 3 from Column 1.
1	61.2	53.9	60.7	-1
2	69.3	59.6	64.7	-7
3	69.8	60.4	70.6	+1

her surface area can be determined from the Du Bois surface area charts by the usual method. If the surface area of the fetus is added to the surface area of the mother thus obtained, and the total calories for each hour are divided by the sum of the surface area of the mother and of the fetus, figures are obtained which represent the heat production of a unit mass of active protoplasmic tissue. The calories for each square meter of body surface thus obtained are plotted as Curve *C* in Charts 1 and 2, and show that there is no essential change in the rate of heat production of a unit mass of active protoplasmic tissue in the human being as a result of pregnancy. The variation in the calories for each square meter of body surface, using the com-

bined surface area of the mother and of the fetus from the Du Bois normal of the mother, 36.5 calories, is plotted as Curve *B* in Charts 1 and 2, and shows fluctuations of only small magnitude, even at the termination of pregnancy. The small mass of the fetus compared to that of the mother allows us to use the standard of the mother without a significant error, as is shown here. The calculation separately of the surface area of the mother and fetus, and the use of the sum of these surface areas to divide into the total calories is correct if, as maintained by Boothby and Sandiford, the relationship between the surface area and basal standard heat production rests on the proposition that the surface area is proportional to the mass of active protoplasmic tissue. Such a calculation would, of course, be invalid if the surface area law depended on Newton's law of cooling. As these authors point out, the latter conception has been abandoned by nearly all users of the surface area method, and the view seems to be very generally accepted that the surface area is the most exact method at present available for estimating the ratio between different individuals of the mass of active protoplasmic tissue.

The validity of this method of calculation can be checked in a very convincing manner as follows: If the average for the 3 days after confinement, of the calories for each square meter each hour (34.9) of the mother is multiplied by the average estimated surface area (1.75) of the mother alone for the 4 days before confinement, the total calories (61.1) of the mother alone just before confinement are obtained. If this figure (61.1) is subtracted from the average of the total calories of mother and fetus for the 4 days before delivery (70.4), the remainder (9.3) may be assumed to represent the total calories of the fetus. Furthermore, if the total calories of the fetus thus calculated are divided by the surface area (0.26) of the baby at birth, which corresponds with the estimated surface area of the fetus for the 4 days before delivery, the heat production of the fetus is shown to be 35.8 calories for each square meter each hour, or 859 calories for 24 hours, which agrees satisfactorily with Talbot's findings (650 to 800 calories) of the metabolism of new-born infants. On the assumption that the heat production of a unit mass of fetal tissue will remain constant throughout fetal life, by multiplying

the heat production for each square meter of body surface each hour by the surface area of the fetus corresponding to its estimated weight, the total calories each hour for the fetus are obtained for the various months. If this latter figure is subtracted from the total calories of mother and fetus, the total calories of the mother alone are obtained, and when divided by the estimated surface area of the mother, the calories for each square meter of body surface of the mother alone are practically the same as those plotted as Curve *B*, obtained by the other method of calculation. Therefore, almost identical figures for the heat production of the mother are obtained by both our methods of calculation, and also the heat production of the fetus, when calculated according to these methods, is what might be expected from the data of Benedict and Talbot. The consistency not only of our results, but of those of other observers, is striking, especially if it is remembered that a very slight variation in the heat production of the mother, because of her relatively large mass, would make absolutely impossible figures for the heat production of the fetus.

Root and Root have reported one case in which the heat production was carefully determined from the 4th month of pregnancy to delivery with occasional rates thereafter, and their findings, as shown in Chart 3, are strikingly similar to ours. The total energy production of the mother was practically unchanged during the early months of pregnancy, but there was a gradual increase beginning with the 8th month, and the peak was reached during the last month. In this particular case, the total heat production had reached its highest level 12 days before confinement, and showed an increase over the basal rate of February 9, of +19 per cent. 3 weeks after delivery the total calories had decreased below the patient's basal rate of February 9, but, as in the case of our subject, this is probably the result of a less active life. Here, again, the fact that lactation is unaccompanied by an increased heat production is significant. If the data of Root and Root are recalculated by our method, the heat production of the mother for each unit mass of active protoplasmic tissue remains unchanged throughout the course of pregnancy, as can be seen by Curves *B* and *C* in Chart 3. If the heat production of the baby is calculated, it is found to be

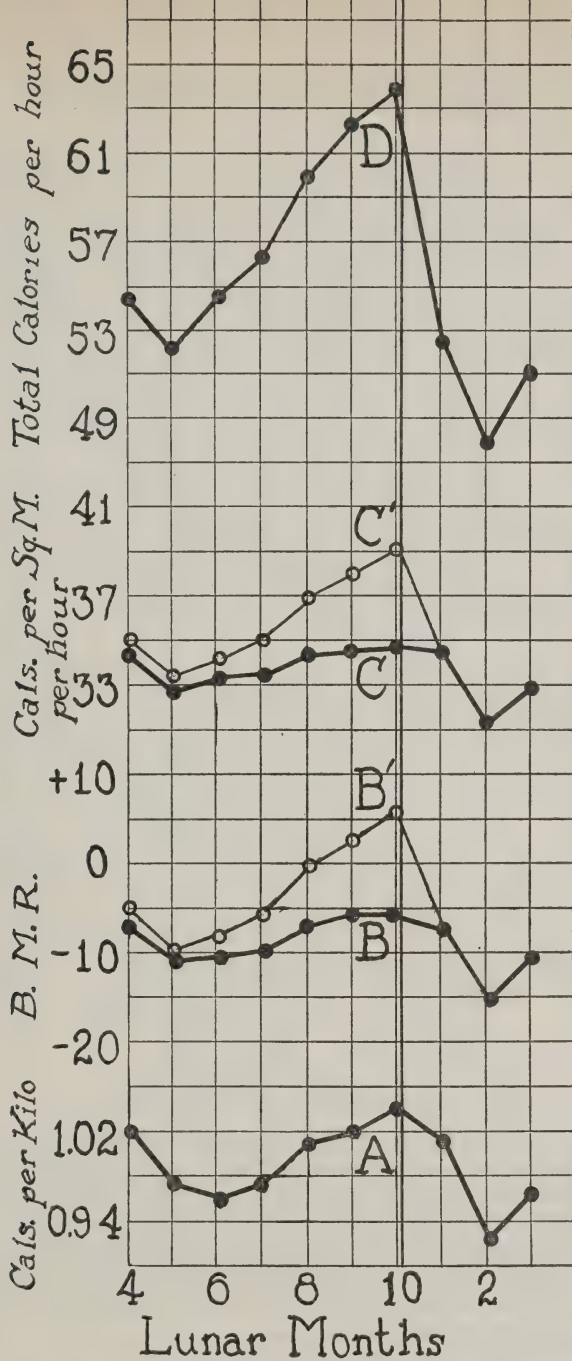


CHART 3. Basal metabolism findings averaged according to lunar months during and after pregnancy. Data of Root and Root. Curve A represents the calories for each kilo; Curves B and C, the basal metabolic rate and calories for each square meter, respectively, calculated according to our method. Curves B' and C' represent the basal metabolic rates and calories for each square meter calculated in the ordinary manner. Curve D represents the total calories for each hour.

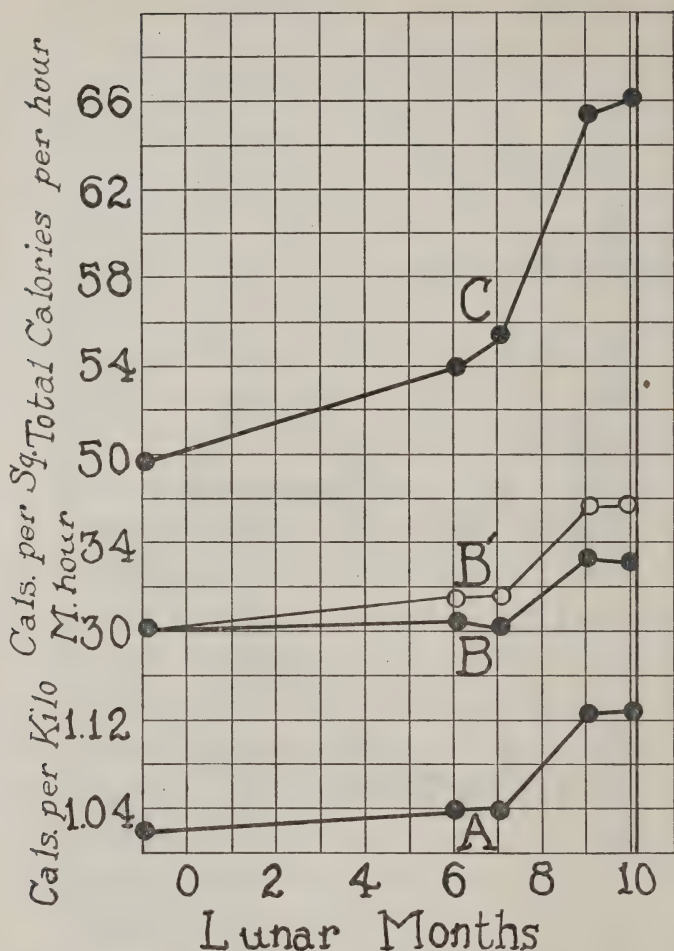


CHART 4. Basal metabolism findings averaged according to lunar months before and during pregnancy. Data on Zuntz's Case B. Curve A represents the calories for each kilo; Curve B, the calories for each square meter, calculating the data according to our method, using the Meeh surface area formula for the mother and the Lissauer formula for the fetus; Curve B' the calories for each square meter, using the Meeh surface area formula and the weight of the pregnant mother in the usual manner. Curve C represents the total calories for each hour.

661 calories for each square meter every 24 hours, a value consistent with that found by Talbot for new-born infants (650 to 800 calories). Joslin found no abnormalities in the metabolism

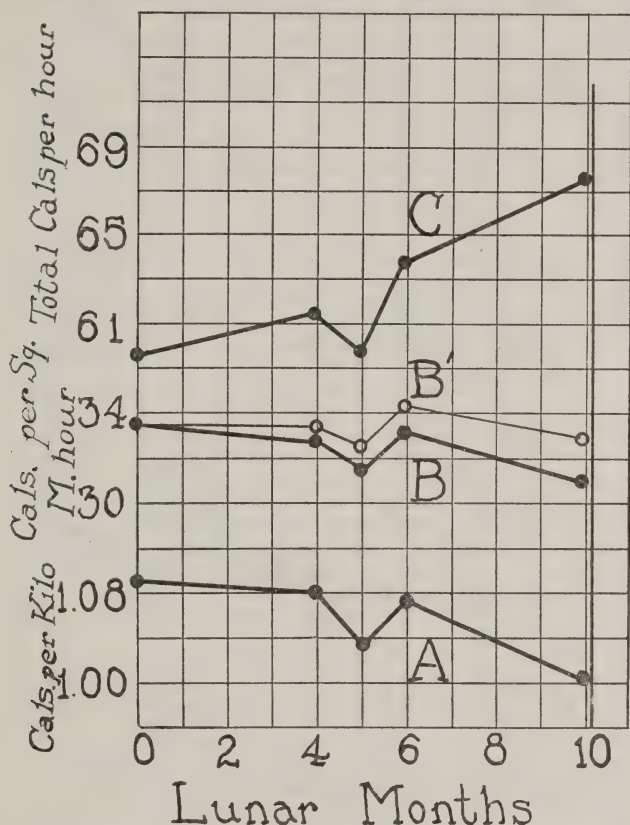


CHART 5. Basal metabolism findings averaged according to lunar months before and during pregnancy. Data on Zuntz's Case C. Curve A represents the calories for each kilo; Curve B, the calories for each square meter for each hour, calculated according to our method, using the Meeh surface area formula for the mother and the Lissauer formula for the fetus. Curve B' represents the calories for each square meter, calculated according to the usual method, using the surface area obtained by Meeh's formula from the weight of the pregnant mother.

of these diabetic patients during pregnancy either as compared with normal women in similar condition or with two patients

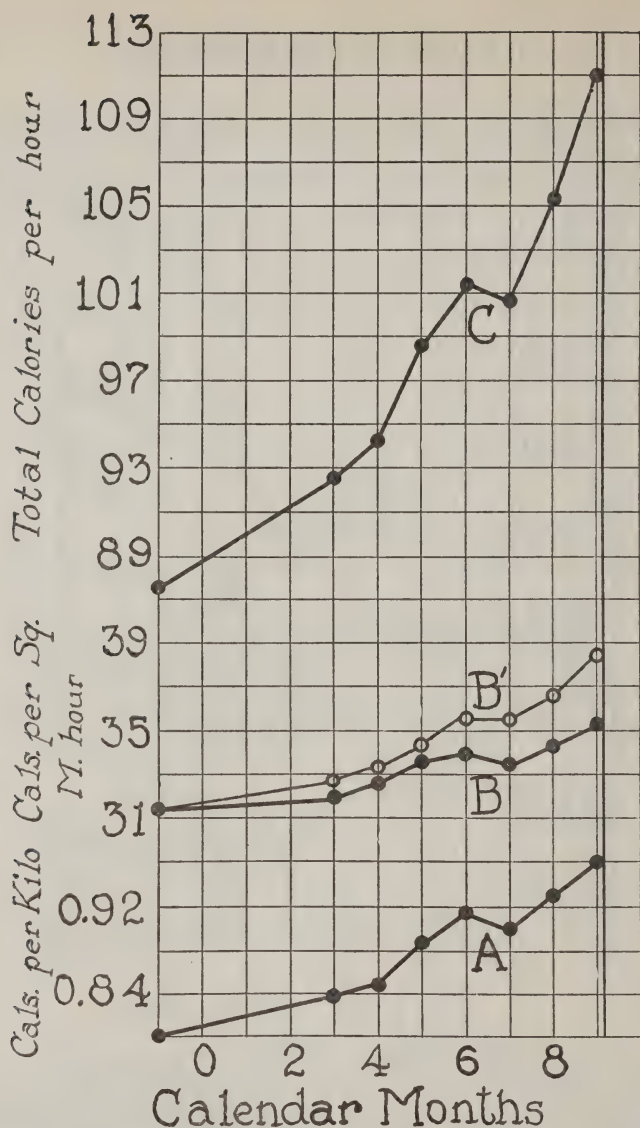


CHART 6. Basal metabolism before and during pregnancy. Data on Magnus-Levy's case averaged according to calendar months. Curve A represents the calories for each kilo; Curve B, the calories for each square meter calculated according to our method, using the surface area of the mother derived by Meeh's formula and the surface area of the fetus by the Lissauer formula. Curve B' represents the surface area derived by the usual method, using the Meeh formula. Curve C represents the total calories for each hour.

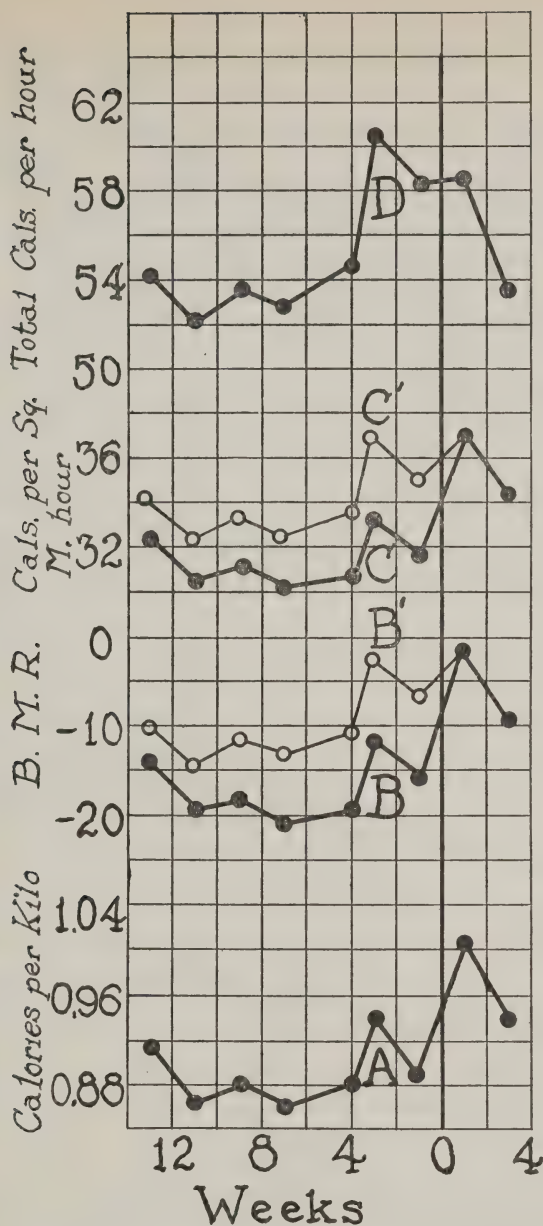


CHART 7. Basal metabolism findings averaged by 2 week intervals during and after pregnancy. Data of Rowe, Alcott, and Mortimer, Case 13, Series II. Curve A represents the calories for each kilo; Curves B and C are, respectively, the basal metabolic rate (Du Bois standards) and calories for each square meter each hour calculated according to our method. Curves B' and C' are, respectively, the basal metabolic rate (Du Bois standards) and calories for each square meter each hour calculated in the usual manner. Curve D is the total calories each hour.

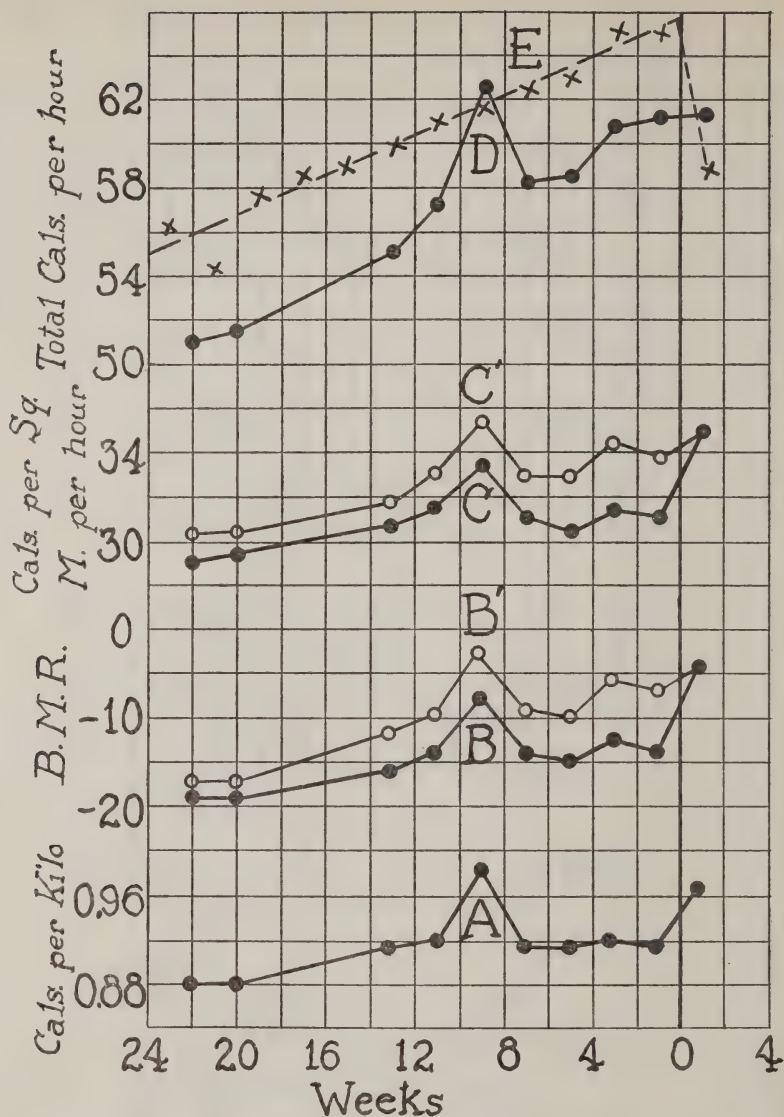


CHART 8. Basal metabolism findings averaged for 2 week intervals during and after pregnancy. Data of Rowe, Alcott, and Mortimer, Case 28, Series I. Curve A represents the calories for each kilo. Curves B and C represent, respectively, the basal metabolic rate (Du Bois standards) and calories for each square meter each hour, calculated according to our method. Curves B' and C' are, respectively, the basal metabolic rate (Du Bois standards) and calories for each square meter each hour calculated according to the usual method. Curve D is the total calories each hour. Curve E represents the average curve for the total calories each hour of the twenty-five cases of Series I of Rowe, Alcott, and Mortimer as given in their Chart 3.

compared with themselves at an earlier period when not pregnant and yet diabetic.

We have also recalculated the data of Zuntz's Cases *B* and *C*, and of Magnus-Levy's case, since these three cases were observed for a considerable time before conception, and during pregnancy. The results are plotted in Charts 4, 5, and 6, respectively, and show a very definite increase in the total energy production, the maximal occurring during the last month of pregnancy. In one of these cases (Curve *C*, Chart 5), there is apparently a decrease of about 8 per cent in the heat production of a unit mass of protoplasmic tissue, while in two, an increase of 10 or 11 per cent (Curve *C*, Charts 4 and 6) is apparent at the termination of pregnancy. However, the effect of fetal movements must be seriously considered as producing a greater part, if not all of this increase, because, as can be seen by a detailed study of the observations in our case, there was a marked increase in total heat production whenever there were noticeable fetal movements during the period of observation. Furthermore, as the height of the subjects of Zuntz and of Magnus-Levy is not known, it was necessary to resort to Meeh's surface area formula instead of the Du Bois, which may also in part account for the apparent small variation in these two cases.

We have had the privilege of reading, through the courtesy of Dr. Rowe, the manuscript of Rowe, Alcott, and Mortimer on the basal metabolism in pregnancy. We have recalculated their data for the two cases presented by them in detail (Case 13, Series II, and Case 28, Series I) and charted them in Charts 7 and 8, respectively. Their data show that the total heat production is increased during the last months of pregnancy, but that, if allowance is made for the surface area of the fetus according to our method, there is little or no change in the rate of heat production of the mother's tissue during pregnancy. We have also plotted in Chart 8 their curve for total calories each hour (Curve *E*), representing the average of the twenty-five cases of their Series I. It is significant that this average curve, although somewhat less steep, nearly parallels the corresponding curve (Curve *D*) of their own cases, as well as of the other cases here presented, and this similarity suggests that, if the data were available for recalculation according to our method, no change

would be found for the average of the twenty-five cases in the total heat production of a unit mass of the mother's tissue during pregnancy.

In Table IV we have recalculated and summarized the data on the one patient reported by Hasselbalch and on Case A reported by Zuntz, both of which were studied during the last month of pregnancy. Hasselbalch's control period was obtained 1 month after delivery, while Zuntz used for comparison a few determinations made in the year previous to pregnancy. These cases, as well as the others referred to, confirm the findings in our own case, as when calculated by our method, no alteration

TABLE IV.
Heat Production of Hasselbalch's Case and of Zuntz' Case A.

Author.	1 Average total calories each hr. of mother in control period.	2 Average calories for each sq. m. each hr. of mother in control period (Meeh).	3 Average total calories each hr. of mother and fetus just be- fore delivery.	4 Average calories for each sq. m. of pregnant mother (Meeh).	5 Percentage variation of Col- umn 2 from Column 4.	6 Average calories for each sq. m. of mother and fetus com- bined (our method).	7 Percentage variation of Col- umn 2 from Column 6).
Hasselbalch.....	55.5*	27.6*	65.1	29.6	+7	27.3	-1
Zuntz, Case A.....	51.6†	30.4†	56.2	33.9	+12	29.9	-2

* Determined 1 month after delivery.

† Determined in year previous to pregnancy.

is found in the rate of heat production in the mother, of a unit mass of active protoplasmic tissue. The slight variations found in the different cases, as well as in the different periods in each case, are within the limits of experimental error. In this regard the statement of Du Bois is pertinent: "We can rest assured that the actual variations of the basal metabolism are smaller than the variations of the measurements."

CONCLUSIONS.

1. Complete data are given on the respiratory metabolism of a normal woman before, during, and for 6 lunar months after pregnancy. The data show, in agreement with those of other investigators, that there is a definite increase in the total heat production during the latter part of pregnancy, in this case beginning with the 8th month and gradually increasing to the 10th lunar month, when the total calories for each hour were 25 per cent greater than before conception.

2. Calculations for this case, as well as for cases from the literature, indicate that the energy production of a unit mass of the mother's protoplasmic tissue remains unchanged throughout the course of pregnancy, and that such increases in the total heat production as occur are due to the increasing mass of active protoplasmic tissue, consisting in large part of the fetal tissues and in lesser part of maternal structures.

3. There was no increase in heat production during lactation. In fact there was a slight decrease, probably because of the less active life. As the mother gradually resumed her usual activities, the metabolism became almost identical with that before pregnancy. There was no demonstrable loss of energy in transforming the mother's food into milk.

4. There were slight daily irregular variations in the heat production after the reestablishment of menstruation, but none which can be accredited to its influence.

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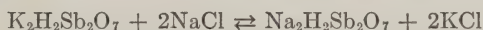
AN IODOMETRIC METHOD FOR THE DETERMINATION OF SODIUM IN SMALL AMOUNTS OF SERUM.*

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Several years ago one of us described a gravimetric method for the determination of small amounts of sodium in ashed serum (1). In this method the sodium is precipitated as the pyroantimonate



and the precipitate dried and weighed. Subsequently Kramer and Tisdall (2) found that the proteins of the serum do not interfere with the precipitation so that it is possible to eliminate the ashing. In this and in several other laboratories satisfactory results have been obtained with these methods. However, other investigators have not been so fortunate. We have always felt that the source of error must lie for the most part in the filtration. The precipitate is finely crystalline and is retained only by very dense filters. It is quite soluble in water and not altogether insoluble even in 30 per cent alcohol. The drying of the precipitate is carried out at 120° C. and the length of time required for the best results seems to vary with the amount of precipitate that has already accumulated on the filter. When the ash of serum is used it must be made alkaline before adding the precipitating reagent. Substances that are insoluble in alkaline solutions are precipitated and weighed with the sodium pyroantimonate. With serum or plasma the error thereby introduced is small, but with urine or stools a preliminary removal of such material is essential for accurate results.

*After submitting this paper for publication we received the August number of the *Biochemische Zeitschrift* in which Bálint (Bálint, M., *Biochem. Z.*, 1924, cl, 424) describes an iodometric method for the determination of small amounts of sodium in simple solutions of sodium salts.

It occurred to us that the method might be further improved by substituting for the gravimetric determination, an iodometric titration of the antimony in the precipitate or determining the residual antimony in the supernatant fluid and thereby indirectly the sodium. We favored the latter procedure at first because it rendered unnecessary the quantitative separation of the precipitate, thereby avoiding errors due to filtration and washing. In order to titrate the antimony in the supernatant fluid, the protein must be removed by a preliminary ashing. To avoid this step we were compelled to return to the determination of antimony in the precipitate. At the suggestion of Dr. A. T. Shohl we removed the supernatant fluid almost completely after centrifuging and in order to effect a complete separation of serum proteins, added a single washing with 30 per cent alcohol. The method as now carried out is the following.

Method.

2 cc. of serum (or the ash of an equal amount of serum dissolved in 2 cc. of 0.1 N hydrochloric acid and made alkaline with 4 drops of 1.8 N alcohol-washed potassium hydroxide) are placed in a 50 cc. tapering, graduated, Pyrex centrifuge tube which is preferably coated with a thin layer of paraffin.¹ 10 cc. of pyroantimonate reagent are added and then exactly 3 cc. of 95 per cent alcohol redistilled over KOH are added drop by drop while stirring with a rubber-tipped glass rod. The tube is stoppered with a cork and allowed to stand for 30 minutes and then centrifuged for 5 minutes. All but 2 cc. of the supernatant fluid is siphoned off. 10 cc. of 30 per cent alcohol are then added and mixed with the supernatant fluid and the sample is again centrifuged. All of the supernatant fluid possible is then removed by means of a suitable pipette and rubber bulb.

Determination of Antimony in the Precipitate.

5 cc. of 10 N hydrochloric acid (concentrated HCl acid, sp. gr. 1.182) are added to the precipitate and the solution of the precipitate is facilitated by stirring thoroughly with a glass rod. The

¹ Unparaffined tubes of Pyrex or "Exax" glass give equally good results. With unparaffined tubes the precipitate tends to stick to the sides of the tubes.

material is then transferred to a 250 cc. Pyrex beaker, tall form, and transference completed by washing with not more than 10 cc. of distilled water. If the beaker is stirred the material will dissolve completely. At this stage a 15 cc. burette, graduated in 0.02 cc., is filled to the zero mark with 0.1 N sodium thiosulfate. 2.0 cc. of a 20 per cent potassium iodide solution are added to the sample. Free iodine is at once liberated and colors the solution a reddish brown and the specimen is immediately titrated with thiosulfate. The latter should be added very rapidly with constant stirring until the brown color is practically gone. 0.5 cc. of a 1 per cent freshly prepared starch solution is added and the titration continued as before until the sample turns brown when further addition of thiosulfate should be made very slowly with thorough mixing between additions. The end-point is reached when the solution becomes water-clear.

Standardization of the Sodium Thiosulfate.

Approximately 5 gm. of potassium iodide are dissolved in 5 cc. of water. This is poured into a 1 liter Erlenmeyer flask and 10 cc. of 3.33 N hydrochloric acid (concentrated hydrochloric acid 1 part, water 2 parts) are added, followed by exactly 50 cc. of the potassium biiodate solution and 180 cc. of distilled water. The sample is rapidly titrated with 0.1 N sodium thiosulfate until the brown color disappears, when 1 cc. of 1 per cent starch solution is added and the titration continued until the almost black color begins to turn a purple. The addition of sodium thiosulfate is continued with caution drop by drop until the sample becomes water-clear. This is the end-point. The volume of potassium biiodate used (50 cc.) divided by the number of cc. of sodium thiosulfate gives the thiosulfate factor. Thus if 50 cc. of 0.1 N potassium biiodate solution are used and 51 cc. of sodium thiosulfate are required to decolorize the solution, the thiosulfate factor will be 0.98.

Calculations.

Calculation When Antimony Is Determined on the Precipitate.

Since 1 equivalent of iodine is freed by the amount of antimony bound to 0.5 equivalent of Na (see discussion below), each cc. of 0.1 N thiosulfate is equivalent to $\frac{2 \cdot 3}{2} = 1.15$ mg. of Na. Hence

(No. of cc. of thiosulfate used) \times (thiosulfate factor) $\times 1.15 \times \frac{1.00}{2} =$ mg. of sodium per 100 cc. of serum or solution when 2 cc. of serum are used. Thus the titration in our analysis was 6.00 cc. of thiosulfate. The thiosulfate factor was 0.96. We have therefore:

$$6.00 \times 0.96 \times 0.192 \times 0.5 \times 100 = 331 \text{ mg. sodium per 100 cc. of serum or solution.}$$

Preparation of Reagents.

Potassium Pyroantimonate Reagent.—500 cc. of distilled water are heated to boiling in a Pyrex flask and approximately 10 gm. of potassium pyroantimonate (J. T. Baker) are added. The boiling is continued from 3 to 5 minutes, the flask immediately cooled under running water, and when the contents are cold 15 cc. of 10 per cent KOH (alcohol-washed) are added. The reagent is then filtered through ash-free filter paper into a paraffined bottle. We have found that frequently some of the undissolved potassium pyroantimonate will pass through even the best filter paper. If the reagent is allowed to stand 24 hours after filtering, all the undissolved potassium pyroantimonate will settle to the bottom. The supernatant fluid is then clear and may be used as long as it remains so. The reagent keeps perfectly well at room temperature for at least 1 month. 10 cc. of this reagent will precipitate 11 mg. of sodium. The 10 per cent KOH should be kept in a paraffined bottle.

Before the reagent is used for the first time, it should be tested for the presence of sodium and also the fact ascertained that none of the potassium pyroantimonate is precipitated by the addition of alcohol in the proportion used in the method. This is accomplished by adding to 10 cc. of the reagent, 2 cc. of distilled water and 3 cc. of 95 per cent alcohol.

When ready the reagent should have a reaction of approximately pH 9 and should contain between 63 and 73 mg. of antimony. After 24 hours the reagent is quite permanent, even when kept at room temperature. 95 per cent redistilled alcohol should be used.

Preparation of 0.1 N Sodium Thiosulfate.—24.822 gm. of sodium thiosulfate are dissolved in 1 liter of water.

Starch Solution.—1 gm. of soluble starch is suspended in 100 cc. of cold water and this heated until the starch goes completely into solution, giving a water-clear solution. This starch solution does not keep and should, therefore, be made fresh each time.

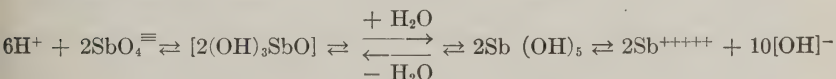
20 Per Cent Potassium Iodide.—200 gm. of potassium iodide are dissolved in a liter of water. The solution turns slightly yellow on standing.

Potassium Biiodate.—3.2946 gm. of "Kahlbaum, best grade" potassium biiodate are dissolved in 1 liter of water. This is used for the standardization of sodium thiosulfate. The solution keeps in glass at room temperature for at least 1 month.

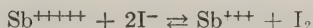
DISCUSSION.

This method involves several distinct steps: first, the precipitation of the sodium as the pyroantimonate compound; second, the separation of the supernatant fluid from the precipitate by centrifuging, followed by either siphoning or careful aspiration of all the supernatant fluid; third, the solution of the precipitate by means of an excess of *concentrated* hydrochloric acid or the acidification of the supernatant fluid with resolution of the precipitate that forms; fourth, the reduction of antimonie ion with a simultaneous oxidation of the iodide ion of hydriodic acid to free iodine; and lastly, the reduction of free iodine to iodide ion by sodium thiosulfate with the formation of sodium iodide and sodium tetrathionate.

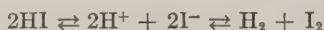
When concentrated hydrochloric acid is added in excess to sodium or potassium pyroantimonate, it forms antimonie acid and potassium or sodium chloride.



In the presence of an excess of H^+ ions this acid probably conducts itself in the manner similar to arsenic acid; that is, it takes up water and ionizes as a base, yielding antimonie ions and (OH^-) ions. The antimonie ions oxidize iodide ions to free iodine and are in turn reduced.



We have been unable to find any data as to the quantitative relationships in this oxidation-reduction reaction, but it goes on in acid solution with great rapidity to completion. Qualitatively it must be similar to the one where ferric ion is reduced to ferrous ion in acid solution by iodide ion. Free iodine is again readily reduced by sodium thiosulfate to iodide ion, but in the presence of oxygen and even in its absence the iodide ion shows a very definite tendency to reform free iodine.² In acid solutions potassium iodide is, of course, converted into hydriodic acid. The H^+ ions of this acid tend to be reduced to molecular H and iodide ions are in turn oxidized to free iodine.



Furthermore iodide ion reacts with the oxygen in the air and water to form free iodine and OH ions according to the equation:



Expressing this equation in the form of the mass law we obtain at equilibrium

$$\frac{(I^-)^4 \times O_2}{(I_2)^2 \times (OH)^4} = K, \text{ equilibrium} = 4 \times 10^9$$

If the iodide concentration = 1 molal, the O_2 , 20 per cent by volume of the air, the temperature, 25° , and (OH) ion concentration, 1×10^{-14} , the concentration of free iodine at equilibrium will be 10^{22} mols. This indicates the tremendous tendency for the formation of free iodine in acid solution and in the presence of oxygen. The equilibrium constants for the two reactions, namely the conversion of iodide ion to free iodine and of oxygen to OH ions, are known only approximately, but with sufficient accuracy to indicate, as Stieglitz has calculated (3), that in the presence of atmospheric oxygen, iodide ion of a molar solution of hydriodic acid would continue to form iodine until a concentration of 10^{22} mols of free iodine is reached. This indicates clearly that an increase of acidity, of the concentration of iodide or of the oxygen tension, will facilitate the conversion of iodide ion to free iodine, whereas an increase of OH ions even in the presence of only a

² This tendency is less marked when the precipitate is titrated.

TABLE I.

Sodium Determinations on a Known Solution of Sodium Chloride.

Sodium per 100 cc. of solution.	
Found.	Present.
<i>mg.</i>	<i>mg.</i>
316	313
318	
318	
313	
311	
316	

TABLE II.

Sodium Determinations on 2 Cc. Samples of a Solution Containing Sodium 3.30 Gm. (0.143 Molal), Potassium 0.237 Gm. (0.00608 Molal), Calcium 0.107 Gm. (0.0025 Molal), Phosphorus 0.190 Gm. (0.0061 Molal), Dissolved in 1 Liter of 0.1 N Hydrochloric Acid.

Found.	Present.
<i>mg. per 100 cc.</i>	<i>mg.</i>
335	330
335	
335	
338	
329	
325	

TABLE III.

Comparative Determinations of Sodium in Ashed and Unashed Serum.

Sodium in unashed serum.	Sodium in ashed serum.
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
324	327
326	327
328	327
333	338
336	340
324	329

trace of free iodine will prevent any further oxidation of iodide ion to iodine.³ The reformation of free iodine can be prevented if the solution be made neutral or alkaline, but in such a solution antimony exists as a negatively charged pyroantimonate ion. By using only a small excess of potassium iodide and titrating rapidly to the end-point, the reformation of free iodine during the titration is reduced to a minimum.

Table I gives an idea as to the degree of accuracy of the method when used for the estimation of sodium in solutions of sodium chloride containing sodium in approximately the same concentration as serum.

Table II gives the results of analyses made on solutions containing, besides sodium, the other inorganic components of serum.

Table III is a comparative study of the same method applied to ashed and unashed serum.

CONCLUSIONS.

An iodometric method for the determination of sodium in small amounts of serum, both ashed and unashed, has been described.

This method has a maximum error of plus or minus 2 per cent. The various sources of error have been discussed.

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2. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 467.
3. Stieglitz, J., *The elements of qualitative chemical analysis*, New York, 1916, i, 272, 284, 306.

³ Neutral solutions of potassium iodide acquire a faint yellow only on prolonged standing at room temperature due to the formation of ions of free iodine. This reaction is accelerated when the solution is acidified.

ON THE DETERMINATION OF SUGAR IN SMALL AMOUNTS OF BLOOD.

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(Received for publication, October 10, 1924.)

A number of modifications of the Folin-Wu method for the determination of blood sugar, adapting it to very small samples of blood, have been suggested.

The majority of these depend, for the initial measurement of the sample, on pipettes of very small capacity, generally of 0.1 or 0.2 ml. size.

Several milliliters can be measured out with a pipette fairly accurately, but when the quantity becomes less than 1 ml., pipetting may easily involve considerable error.

One of the advantages claimed for the micro methods is that the small volume of blood required may be taken from the tip of the finger. This necessitates depending on capillary force, and, therefore, the bore of the pipette must be made rather narrow. The ratio of the surface of contact between liquid and glass to the volume of sample is therefore large. Blood is a viscous fluid, subject to clotting. In view of these conditions results so obtained must be considered of doubtful value.

Measurements made with micro pipettes yielded results which go to substantiate this claim. Three 0.1 ml. pipettes were first standardized with water, then with blood, and with sodium chloride solution of the same specific gravity. As seen from Table I, there is considerable variation among the individual measurements and also from the theoretical.

The present method, in brief, measures the sample by weighing on a torsion balance, and the sugar is determined by a modification of the Folin-Wu procedure adapted to small amounts of blood.

Apparatus.

To obviate the possible errors of volumetric measurement in the present method, samples are measured gravimetrically and since weighing on the ordinary analytical balance is a comparatively lengthy procedure and would therefore necessitate an anticoagulant, an accurate torsion balance is used. A description of this balance may be found in Ivar Bang's¹ "Mikromethoden zur

TABLE I.

	Blood (sp. gr. 1.055).	Sodium chloride solution (sp. gr. 1.055).
	<i>gm.</i>	<i>gm.</i>
Pipette E (0.180 ml.).	0.1120	0.1167
	0.1101	0.1134
	0.1112	0.1162
	0.1157	0.1143
	Theory . . . 0.1139	
Pipette W (0.0976 ml.).	0.0980	0.1010
	0.0976	0.1028
	0.0956	0.1003
	0.0976	0.1009
	Theory . . . 0.1030	
Pipette R (0.0974 ml.).	0.0992	0.1048
	0.0945	0.1014
	0.1004	0.1028
	0.0934	0.1039
	Theory . . . 0.1028	

Blutuntersuchung," and in Abderhalden's² "Handbuch der biologischen Arbeitsmethoden." The torsion balance used in this work was made by Gallenkamp and Co.,³ and has a capacity of 500 mg. with a sensitivity of 0.5 mg.

A method was devised for weighing the samples in small glass tubes which can be made in any laboratory very quickly and

¹ Bang, I., Mikromethoden zur Blutuntersuchung, Munich, 1922.

² Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin, 1921, 1. Abt., pt. 3, 252.

³ Gallenkamp and Co., 19 Sun St., London E. C. 2, England.

cheaply. The shape of the tubes is readily seen from Fig. 1. They are drawn from ordinary test-tubes. These are first thoroughly cleansed with chromic acid and rinsed with distilled water. Then a portion of about 1.5 to 2 inches is heated in the flame of a Meker burner and, when the walls are beginning to collapse, drawn out fairly rapidly to a diameter of 3 to 4 mm.

Since the glass is now very thin, the tips must be drawn with considerable care. The lowest possible yellow Bunsen flame is best. The drawn tube, about 5 to 7 mm. in length, is cautiously

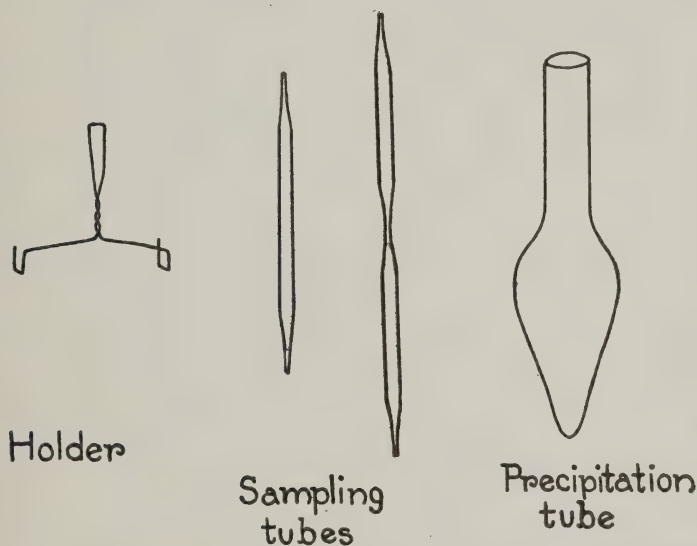


FIG. 1.

heated in the small flame and, as soon as the glass softens, drawn to make the outside diameter of the tube about 1 mm. The tubes should be 40 to 50 mm. in length. Owing to the thinness of the walls a very sharp file must be used in cutting the tubes apart. In general from twenty-five to thirty tubes are obtained from one test-tube. The tips are fire-polished by cautiously bringing them near a small flame.

The making of the tubes requires so little time, effort, and material, that it has not been found worth while to clean the tubes for further sampling. They have been thrown away after use. If desired, they can easily be cleaned and made ready for further

work by immersing them for a time in a weak ammonia solution and then successively aspirating through them distilled water, alcohol, ether, and air.

These tubes, of course, can only be used when the sampling is done in the immediate neighborhood of the balance, a limitation which practically applies to the original Bang method of sampling as well. A special tube has been devised, if for some reason it is necessary to do the sampling at some distance from the balance. In this type of tube the size cannot vary by wide limits, but must be such that one of the sections of the tube holds about the amount of sample desired, from 0.1200 to 0.2000 of a gram. Previous to sampling, the tube is placed in a mixture of sodium fluoride and thymol, recommended by Sander as anticoagulant and preservative, especially against glycolysis, and slight suction applied, whereby a small amount is readily drawn up into the tube and is deposited at the constriction at the middle of the tube. An amount of preservative, equal to 1 per cent of the weight of sample, is recommended, though it is stated 10 per cent will not affect the results. About 3 mg. is a good quantity to use. Until the tube is used it is best to close both tips with small blocks of pencil eraser rubber, so that no thymol fluoride can be lost. It must be kept in a wrapper on which the weight is marked. The sample, when drawn, must fill the tube containing the preservative from the tip well into the middle constriction. A piece of catheter tubing No. 9, which has an inside diameter of 1.5 mm. and an outside diameter of 3 mm., is attached at the other end and the blood slowly drawn through the constriction well into the empty portion. It is then slowly blown back and in this way passed back and forth until all the blood is mixed with the anticoagulant. The tube is again placed between the small blocks of rubber and can now be kept in a cool place for several days without apparent loss in sugar content. After the tube is weighed with the sample, the manipulation is the same as with the other tubes.

The holder for the tubes is made of twenty-seven or twenty-eight B and S gage brass spring wire, which has a diameter of 0.013 mm. Its construction is simple and easily understood from Fig. 1. A piece of the wire, about 10 cm. long, is twisted at the middle to form a rather long loop and the ends are bent to form stirrups about 2.5 or 3 cm. apart. The holder weighs 60 to 70 mg.

The shape of the precipitating tubes is best understood from Fig. 1. The bulb is of such a size that when filled with 4 ml. of fluid, the meniscus is in the neck, a little above the bulb. The lower portion of the Folin-Wu boiling tubes serves very well. The inside diameter of the neck should not exceed 6 mm. The tube is carefully graduated at 4 ml.

A home-made pipette for washing out the sampling tubes is required. This is a piece of ordinary 6 mm. glass tubing about 20 cm. long. The glass at the tip should be rather heavy to prevent undue breakage.

3 ml. Ostwald pipettes are used for transferring aliquot portions of the samples to the boiling tubes. It must, however, be noted that the tip should be of such length that the pipette readily fits through the neck of the precipitation tube nearly to its bottom.

The tubes for making the reduction are of the usual Folin pattern, the lower part being an exact duplicate. 4 ml. of liquid completely fill the bulb and part of the narrow portion. The upper part of the tube is narrower. It has an inside diameter of 13 mm. and is graduated to hold 12.5 ml. The length of the entire tube is the same as that of the larger tube. The reagents employed are the same as in the macro method except for the alkaline copper solution and the sugar standards.

It was desired to keep the relative concentrations of the reacting substances, as well as the total volume, the same as in the macro method. For accuracy, it was advisable to keep the volumes measured, and the aliquot of sample taken, as large as possible. The concentration of the alkaline copper reagent was therefore doubled, making only 1 ml. of this reagent necessary, and permitting a corresponding increase in the volume of blood filtrate used in the reduction. It is made up as follows:

Dissolve 80 gm. of pure anhydrous sodium carbonate in about 600 ml. of water and transfer to a liter flask. Add 15 gm. of tartaric acid and when solution is complete, add 9.0 gm. of crystalline copper sulfate. Make up to a volume of 1 liter.

To prepare the weaker sugar standard dissolve 33.3 mg. of pure glucose in a saturated (about 3 per cent) solution of benzoic acid and make up to 1 liter in a volumetric flask. 3 ml. of this solution will contain 0.1 mg. For the stronger standard dissolve 66.7 mg. of glucose in saturated benzoic acid and make up to 1 liter.

Procedure.

A precipitation tube containing about 0.5 ml. of water, a piece of catheter tubing about 4 cm. in length, and a small beaker with distilled water are kept at hand.

The sample is taken as follows: A sampling tube is first weighed. The best place to take the blood is at the inside of the thumb near the nail. The part is cleaned with alcohol and an incision made with a spring or ordinary lancet. The first drop of blood is removed with a little absorbent cotton and then the tip of the weighing tube touched to the wound. The capillary attraction immediately draws the blood up and as more drops appear, the tip of the tube is applied until the necessary amount, normally between 120 and 180 mg., has been taken up. The finger is best held in such a position that the blood runs down into the tube, but not at too great an inclination from the horizontal, otherwise there would be a tendency for the blood to run to the other end of the tube, and while that does not cause the loss of the sample, it does prevent further taking up of blood. The tube is now reweighed quickly; a piece of catheter tubing is attached to that tip of the sampling tube which is free of blood and the sample blown into the water contained in the precipitating tube. With a little practice, the entire procedure, that is, the drawing of the sample, weighing, and transferring to a precipitating tube, does not take more than $\frac{1}{2}$ to $\frac{3}{4}$ of a minute and no trouble is experienced from clotting.

The mixture is shaken and drawn into the sampling tube several times. For rinsing, the sampling tube is still held in the precipitating vessel, but not dipping into the liquid. A little distilled water, 0.2 or 0.3 of a milliliter, is drawn into the washing pipette, its tip inserted into the catheter tubing, and the water blown gently through, thereby washing the sampling tube. When this has been repeated once or twice, the outside of the tube near the tip is rinsed by means of the washing pipette. The inside of the tube may now be rinsed twice more and it will then be entirely free of blood. The amount of liquid in the precipitating tube will be about 1.5 ml. If it is desired, duplicates may readily be taken, but then two persons are required, as the first sample must be weighed while the second is being taken. Generally the subject, if not too nervous, can draw the second sample, as the technique is very simple.

The tube is shaken to insure homogeneity and to hasten laking. The precipitating agents are now added. By means of 1 ml. pipettes, graduated in hundredths, 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid are added, the amount of each in milliliters being equal to the weight in grams of the sample increased by 0.02. That is, if the sample weighed 0.1235 gm., 0.14 ml. of tungstate solution and the same amount of acid would be used. The small excess of precipitating agent does no harm and insures complete precipitation. The mixture is thoroughly agitated by rolling the tube between the palms of the hands and then allowed to stand for 5 minutes. It is now carefully made up to the 4 ml. mark with water, closed, and thoroughly shaken.

The tube is whirled in a high speed centrifuge for 3 minutes. By means of the Ostwald pipette 3 ml. of the clear supernatant liquid are drawn off. Since the precipitate occupies a volume of 0.5 ml., this amount of clear liquid is readily available. The protein is, however, easily stirred up if suction is not continuous or if the tip of the pipette touches the sediment. Since it is desirable to see the meniscus of the liquid in the pipette during the operation, the aliquot portion is conveniently taken before a mirror. In this way the tip of the pipette may be easily seen and the position of the meniscus in the pipette is also readily observed.

The 3 ml. of sample are transferred to a modified Folin tube and 1 ml. of the double strength alkaline copper solution is added. From this point on, the manipulation is exactly as in the macro method. A blank test should frequently be run on the alkaline copper solution, that is, the usual amount diluted with water instead of sugar solution to 4 ml. in the boiling tube, boiled as usual, and then the molybdate phosphate solution added. Color equivalent to several hundredths per cent sugar has been obtained from alkaline copper solutions as used in the macro method. These solutions gave no color when molybdate phosphate solution was added without previous boiling and, while one contained a slight sediment of cuprous oxide, another, which gave a color equivalent to 0.025 per cent sugar figured on a 0.1 gm. sample, had no sediment whatever. The double strength alkaline copper solution used in the present work gave so slight a color that its equivalent in terms of sugar could not be measured. Throughout this work a Bock-Benedict colorimeter was used.

A series of comparisons of the macro and micro methods was run on samples of preserved blood (Table II). The micro results given in this table have all been calculated to volume percentage by multiplying the weight in percentage by 1.06.

TABLE II.

Type of blood.	New method, per cent by volume.	Folin-Wu method, per cent by volume.
	<i>per cent</i>	<i>per cent</i>
Beef.....	0.071	0.068
"	0.072	0.068
" *	0.180	0.183
"	0.180	0.181
"	0.237	0.243
"	0.237	0.240
"	0.158	0.157
"	0.156	0.157
"	0.158	0.157
"	0.174	0.177
"	0.176	0.177
"	0.182	0.177
"	0.237	0.235
"	0.237	0.238
"	0.235	0.238
Human.....	0.101	0.097
"	0.123	0.120

* Glucose added.

SUMMARY.

1. A method for the determination of sugar in small amounts of blood is presented. Small glass tubes easily made in the laboratory are used to weigh the samples on the torsion balance. The sugar is determined by a modification of the Folin-Wu procedure.

2. Data are presented to show that pipettes of small capacity are not reliable for measurement of blood samples.

3. Results by the new method agree well with those of the old method.

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DIETARY REQUIREMENTS FOR REPRODUCTION.

III. THE EXISTENCE OF THE REPRODUCTIVE DIETARY COMPLEX (VITAMIN E) IN THE ETHEREAL EXTRACTS OF YELLOW CORN, WHEAT EMBRYO, AND HEMP-SEED.*

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(Received for publication, October 7, 1924.)

In connection with his studies on the rôle of proline in nutrition, begun at the Department of Agricultural Chemistry, University of Wisconsin, in 1918, the author became interested, from work then in progress, in the supplementary value of lactalbumin to casein, fed in the same proportions as these proteins are found to occur in milk. In the series of experiments initiated in the early part of 1919 (1) skimmed milk powder, fed at a 9.6 per cent level, was found to be excellent for growth, but was found, in the presence of a suitable salt mixture and an abundant supply of the known vitamins, to be inadequate for reproduction. The cause for failure was attributed to the possibility that the amino acid requirement of the mammalian organism for the physiological processes of fertility and lactation may be different from the requirement for growth, from the standpoint either of quantity or of quality, and so for 2 years experiments to elucidate the failure of reproduction on diets composed of purified food substances were conducted from the amino acid standpoint. Later on, after the quality of the protein in the diet had been improved, intensive efforts to produce fertility and to obtain success in lactation by increasing the known vitamins in the diet resulted in failure. No attempts were made to improve the nature of the inorganic ion complex of the synthetic diets. In these experiments it was found that the addition of one seed to the pure diets always resulted in fertility and, in a good many cases, fair success in rearing of young was secured.

* Research paper No. 11, Journal Series, University of Arkansas.

The conclusion, which appeared in a previous publication in this Journal, was as follows (2): "In addition to the antixerophthalmic, antirachitic, antiberi-beri, and antiscorbutic vitamins, there exists another hitherto unrecognized vitamin, that is essential for reproduction, which becomes evident only in breeding experiments where rations composed of purified food substances are employed." In that paper a reproductive vitamin was anticipated, although no positive data were then available. In this paper positive evidence is presented showing that ether extracts from yellow corn, wheat embryo, and hemp-seed, an organic factor (which, according to a suggestion formulated in a previous article (2), I choose tentatively to call vitamin E) essential for reproduction.

Recently Anderegg (3) published an article, entitled "Diet in relation to reproduction and rearing of young," in which he asserts that it is not necessary to assume the existence of a specific vitamin for reproduction. Since all Anderegg's diets contained large concentrations of butter fat, his conclusions are untenable, as has been pointed out by Evans (4). With high concentrations of butter fat in the diet Evans and Bishop (5) found that sterile females become pregnant and manifest a certain degree of success in rearing of young.

Since McCollum and Davis (6) and Osborne and Mendel (7) have independently demonstrated that fat-soluble A, or the antixerophthalmic vitamin is present in butter fat and cod liver oil, but is absent in cottonseed, linseed, and olive oils, it occurred to me that the reproductive dietary complex might, by chance, be contained in vegetable oils. This whole paper deals, then, with attempts to secure fertility and to obtain successful lactation with laboratory-prepared oils, and with commercial vegetable oils.

EXPERIMENTAL.

Preparation of the Ethereal Extracts of Wheat Embryo, Yellow Corn, and Hemp-Seed.

Wheat Germ Oil.—The wheat germ oil was extracted in a large Soxhlet apparatus. About 1,000 gm. of wheat embryo were allowed to extract for 7 hours until the solvent siphoned six to eight times. The ether was then distilled off and the residual ether in the wheat oil expelled by blowing a current of air from an electric fan for 3 hours.

Corn Oil.—The corn oil was prepared in a similar manner, only the extraction was allowed to proceed for 14 hours. Later on it was found that acetone is a more efficient solvent for corn oil from the standpoint of quantitative returns, and so another preparation was obtained by extraction for 14 hours with hot acetone.

Hemp-Seed Oil.—The hemp-seed oil was extracted in the cold by allowing crushed seed to stand in contact with large volumes of ether for 24 hours. Most of the ether carrying the oil, which settled on top, was siphoned off, and the residual solvent squeezed out through cheese-cloth. The ether was then distilled off, and the residual solvent expelled by the aid of an electric fan running for 3 hours.

Two series of experiments were carried out: (I), using casein as the basal protein; and (II), using protein derived from skimmed milk powder purchased from the Merrell-Soule Company, New York.

Series I.

Composition of Control Rations (No Vegetable Oil Present).

<i>Ration 441.</i>		<i>Ration 444.</i>	
Casein.....	25.0	Casein.....	25.0
Salts 32.....	4.0	Salts 32.....	4.0
Agar-agar.....	2.0	Agar-agar.....	2.0
Cod liver oil.....	2.0	Cod liver oil.....	2.0
Dextrin ¹	67.0	Harris yeast-vitamin powder.....	0.4
		Dextrin.....	66.6

The publication of Osborne and Wakeman's paper (8) on the extraction from yeast of a very potent water-soluble vitamin B preparation encouraged the use of such a desiccated extract in all this work. Such extracts were purchased from the Harris Laboratories, Tuckahoe, New York, which is producing a standardized product (9).

After being used for 8 weeks, this vitamin preparation, to obtain normal growth, had to be increased to 0.6 per cent, and from the time of breeding until the termination of the experiments it was further increased to 1.0 per cent of the total ration.

In rations of Series I, containing vegetable oils, 5 per cent of the oil employed replaced 5 per cent dextrin.

¹ Dextrin carried an 80 per cent alcoholic extract of 30 gm. of ether-extracted wheat embryo.

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Series II.

Composition of Control Rations (No Vegetable Oil Present).

<i>Ration 465.</i>		<i>Ration 483.</i>	
Skimmed milk powder...	50.0	Skimmed milk powder....	35.0
Ferric citrate.....	0.2	Ferric citrate.....	0.2
Agar-agar.....	2.0	Agar-agar.....	2.0
Harris yeast-vitamin powder.....	0.4	Harris yeast-vitamin powder.....	0.5
Cod liver oil.....	2.0	Cod liver oil.....	2.0
Dextrin.....	45.4	Salts 32.....	1.5
		Casein.....	5.0
		Gelatin.....	5.0
		Dextrin.....	48.8

The composition of the rest of the rations containing laboratory-prepared and commercial vegetable oils is shown in Charts V to XIII inclusive.

After being used for 4 weeks, the yeast vitamin extract in the Series II rations was increased from 0.4 to 0.5 per cent of the total ration.

In all the work reported in this paper all the animals,² with the exception of a few individuals, made normal growth. If an animal did not make normal growth, it was discarded and not used for breeding.

Since normal growth was secured on all rations, it was thought that considerable space can be saved by omitting charts of growth and by showing only charts of the growth of litters. One chart of growth, however, was considered of interest to be inserted—that showing skimmed milk powder as a source of the water-soluble B vitamin. (See Chart IV.)

Two males and three females were used for each experiment. The males and females were placed in separate neighboring compartments and bred when they were from 90 to 100 days old (10). Pregnant females were separated in individual compartments 3 or 4 days before delivery was expected, and were given wooden boxes in which to deposit and rear their litters. If the females had milk, they almost invariably became accustomed to the box and kept their young in it. As soon as milk flow stopped,

² No growth was made on the ration containing fish oil, because of lack of food consumption.

however, the mothers either devoured the young or scattered them on the screens, so that the litter would be found dead in shavings on the bottom of the galvanized pan.

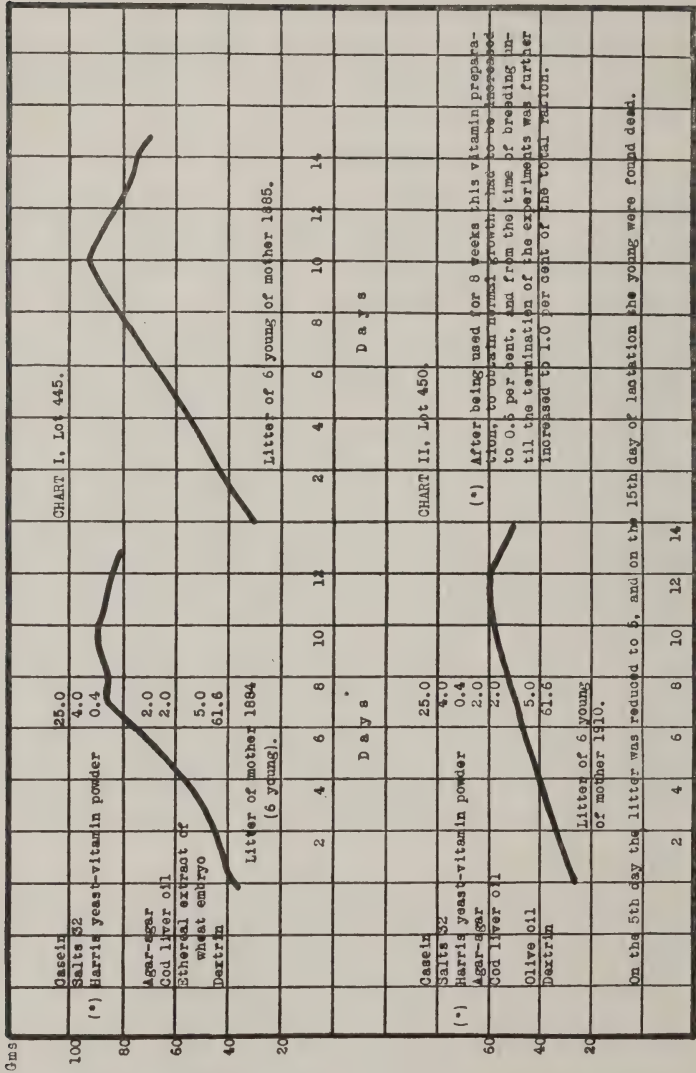
In all cases the litters were reduced to six, so as not to overburden the mammary gland and at the same time provide an exact method of comparison.

DISCUSSION.

When casein was used as the basal protein in Series I experiments, no fertility was obtained on Control Rations 441 and 444, containing no vegetable oils, although normal growth was secured with all animals. On the other hand, 100 per cent fertility was secured with ethereal extracts of the wheat embryo and hempseed. Only one female out of three became pregnant on Ration 450, carrying commercial olive oil. 100 per cent fertility was also obtained with commercial cottonseed oil, but the data on this oil have been omitted in this paper, since the experiments are being continued with skimmed milk powder from the standpoint of lactation and will be published later in connection with work in progress on peanut, soy bean, peach kernel, and sunflower oils. No fertility was obtained on Rations 447 and 451 which contained commercial linseed and cocoanut oils.

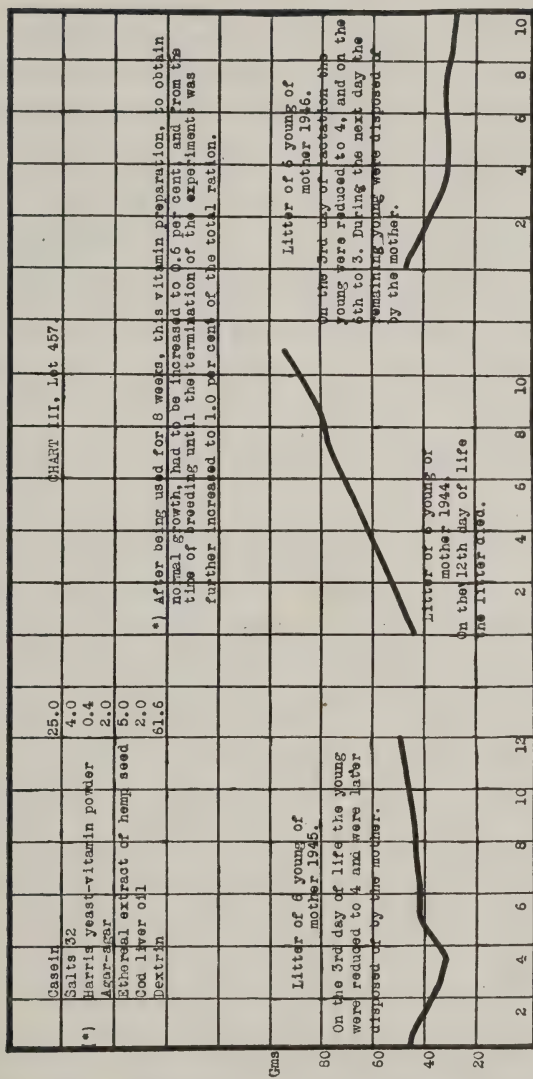
Charts I, II, and III, however, indicate that rearing of young on Series I rations was rather unsuccessful. Two females on Ration 445, which contained a 5 per cent ethereal extract of wheat embryo, reared their litters of six young successfully for periods of 7 and 10 days, after which periods the mammary glands of the mothers became dry, and the young, of course, began to lose weight rapidly.

Recently Evans stated that (4): "wheat embryo, in the fat-free condition, is as effective in its galactogogic action as the whole wheat embryo, and that, it would appear, therefore, that the food material necessary for mammary function is not soluble in fats." In this connection the author would like to state here the results of two experiments out of a series not yet completed, which strongly suggest that the organic factor necessary for reproduction is a fat-soluble vitamin. On a ration containing 20 per cent whole wheat embryo three females have given birth to three normal litters and have reared them successfully through-



CHARTS I AND II.

Days



Days

CHART III.

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out the lactation period. Whole wheat embryo, after being extracted with ether for 7 hours, still contains 0.4 per cent fat. A ration containing 20 per cent of such ether-extracted wheat germ carries 0.08 per cent fat. On such a low fat-containing ration normal litters were born, but the young were born dead. It seems, then, that very small amounts of wheat oil are required for fertility, but considerably more for proper nourishment of the fetus during gestation. The mammary glands of the mothers giving birth to litters on the ether-extracted wheat embryo rations were found completely dry. On a diet containing 35 per cent of ether-extracted wheat embryo two females gave birth to normal litters and nursed them for a few days, but then disposed of them.³ Such results would indicate that the oil as found in the germ of wheat is necessary not only for fertility and proper nourishment of the fetus during gestation, but also for successful lactation. The detailed data of the experiments referred to above will be published in a subsequent communication.

It was anticipated that the most successful results in fertility and lactation would be obtained by employing skimmed milk powder for the following reasons: (1) It contains all the proteins of milk; (2) when fortified with small amounts of ferric citrate, it furnishes a superior salt mixture; (3) when fed at a 50 per cent plane of intake, it in itself serves as an excellent source of the water-soluble B vitamin (see Chart IV); and (4) it is absent in the organic factor (vitamin E) essential for fertility.

During the first 5 to 7 weeks all animals on skimmed milk powder rations suffered with considerable diarrhea, possibly due to gastrointestinal disturbances produced by the large amounts of lactose in the diet (11). During the diarrhea period the animals had a rough coat and looked somewhat abnormal, but their remarkable gains in weight would seem to disprove their apparent ill state of health. After 8 weeks, intestinal troubles ceased and

³ These experiments were completed during May of this year, and the results were communicated before the Arkansas Medical Society of Fayetteville, on May 20, 1924. Series I experiments, using casein as the basal protein, were completed during July of this year. Evans' article on "Unique dietary needs for lactation," which appeared in *Science*, Vol. lx, No. 1540, is dated July 4, 1924.

the animals developed a smooth coat. They made normal growth and were completely resistant towards diarrhea. Not infrequently certain individuals overcame the diuretic effect of their skimmed milk diet 4 weeks after weaning time.

When a litter is born the mother is watched very carefully with respect to her attitude towards the young. When a mother has normal mammary function her maternal instinct guides her

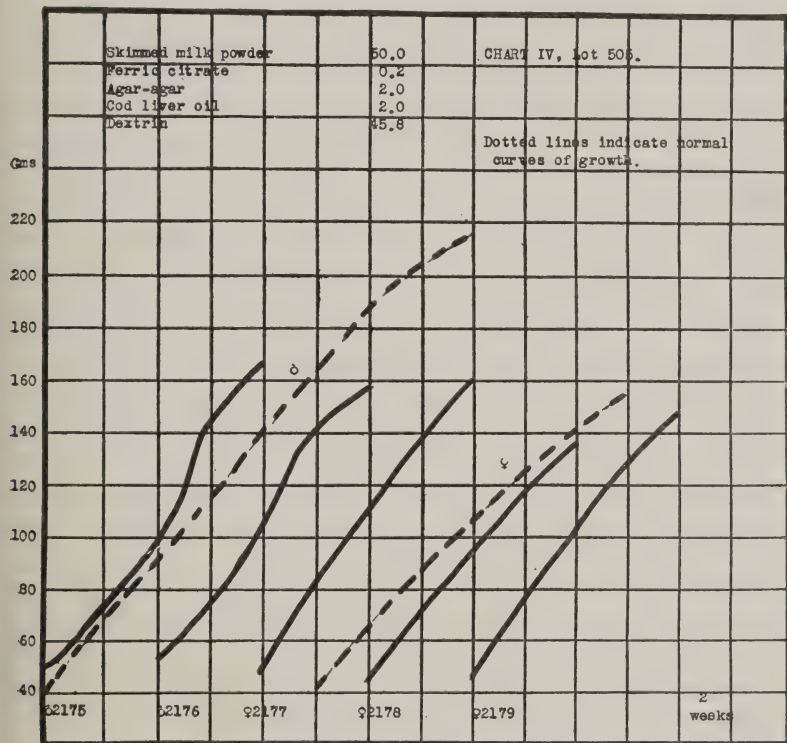


CHART IV.

to be very solicitous towards the welfare of her litter. She stays in the box provided for her, keeps the young warm, and nurses them most of the time except during intervals when she must go to drink and eat her food. When, however, there is lack of milk flow and she feels she is unable to supply nourishment, the mother becomes very irritable and makes no attempt to save her baby rats. She either devours them or scatters them on

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the screen, so that the young are later found cold in the shavings of the galvanized pan below the screen. After the young have been separated from the mother any length of time, they naturally die. Sometimes the mother scatters the young on the platform of the feeding pan and completely ignores them.

On August 19 and 20 seven litters were born to females partaking of skimmed milk powder rations containing ethereal extracts of wheat embryo, yellow corn, and hemp-seed. On the 2nd day after delivery two females on Ration 466, containing 5 per cent of wheat germ oil, were found ignoring their litters and scattering them all over the screens, and all attempts to induce them to take care of their young resulted in failure. On the 2nd day both litters were found dead. One female on Ration 467, containing a 3 per cent ethereal extract of wheat embryo, reduced her litter of six given her to four, and these died on the following day. Fortunately, however, one female on Ration 466, one on Ration 467, one on Ration 468, containing hemp-seed oil, and one mother on Ration 469, containing corn oil, showed evidence of at least a desire to keep their litters. When the litters were weighed, however, on the 2nd and 3rd days of lactation, they showed very little, almost insignificant gains in weight, as indicated in Charts V, VI, and X. Our experience with hundreds of litters of stock rats receiving natural foodstuffs supplemented with a liberal supply of whole milk shows that very frequently there is little milk flow during the first 24 hours of lactation, but that the young invariably gain significantly on the 2nd day of lactation, and that occasionally by the end of the 3rd day and very often on the end of the 4th day of lactation the litters will as much as double their initial weight.

When several mothers had already disposed of their litters and some were maintaining them with little increases in weight, it occurred to me that the requirement of the water-soluble B vitamin for normal mammary gland function may be ever so much greater than the requirement for body growth and stimulation of the appetite. The rôle of the water-soluble B vitamin in lactation had already been pointed out in reproduction studies of Georgia velvet bean pod meal (2). In these experiments it

was found that when dextrin carried 10 gm. of alcoholic extracts of ether-extracted wheat embryo in a 100 gm. ration, litters of four young were reared for about a week. Increasing the concentration of the water-soluble B vitamin to 20 per cent resulted in the rearing of litters of four young for about 13 days. When, however, the dextrin in the ration carried 30 gm. of alcoholic extracts of ether-extracted wheat embryo, introducing a 30 per cent concentration of water-soluble B vitamin, a litter of four young was weaned, although it was not reared normally throughout the lactation period. A similar situation was readily anticipated in these (Series II) experiments when failure in rearing of young seemed inevitable, because of the very small gains in weight of the litters as late as the 3rd day of lactation, and because of the small amounts of milk found in the mammary glands of the mothers. An attempt was then quickly made to improve lactation by increasing the concentration of the water-soluble B vitamin with the potent yeast extract furnished by the Harris yeast-vitamin powder prepared according to the Osborne and Wakeman technique (8), which is a standardized product, from 0.5 to 1.0 per cent of the total ration. At this time the same change was made in all skimmed milk powder rations on which females were bred. While no response was obtained 24 hours after the increase in the water-soluble B vitamin concentration was made, most remarkable gains were made by the litters which previously seemed doomed to failure, 48 and 72 hours after the addition. The mothers began to secrete milk and the most surprising success was secured with all the litters. The young made practically normal growth throughout the period of lactation and were successfully weaned on the 21st to the 25th day after birth.

Evans (4) reports that he can cure sterility with 1 to 6 drops of wheat germ oil daily. Lactation, however, is seriously impaired on "pure" diets. "The average weaning weights of the animals resulting from such lactation is almost exactly half that which is normal, *i.e.*, 20 grams instead of 40 grams on the twenty-first day of life."

Very seldom can a young rat, unless he attains a weight of from 33 to 35 gm., be successfully weaned, because until he becomes that large he is not able to eat food and drink water. It

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would seem, then, that Evans with his pure diets was only partly successful in lactation, since he was not able to wean his litters.

On the skimmed milk powder Rations 465 and 483, which contained no vegetable oils, no fertility was obtained, even after the water-soluble B vitamin concentration was increased by increasing the Harris yeast-vitamin powder, in the case of Ration 465 to 1.0 per cent; and in the case of Diet 483, by fortifying the 0.5 per cent potent yeast extract with an 80 per cent alcoholic extract of ether-extracted wheat embryo to the extent of 30 per cent in the ration. Young of mothers, however, were successfully reared to weaning age on the ethereal extracts of wheat embryo, yellow corn, and hemp-seed, and the young weighed 20 gm. each on the 11th to the 14th day of life.

Food consumption records clearly show that the response obtained to the increase in concentration of the potent yeast extract is not to be attributed to the fact that the mothers ate more after the 4th and 5th days of lactation. The same is true with respect to the controls. In the four control experiments (Rations 441, 444, 465, and 483) all animals ate as much as they did in the experiments in which vegetable oils were incorporated. In the control experiments, however, the females were sterile, while in the latter experiments the presence of vegetable oils led to fertility in certain cases and to fertility and successful weaning of young to the second generation in others.

From records of food consumption, it was calculated that each animal was consuming about 60 mg. of the potent yeast extract per day during breeding, gestation, and lactation periods when the rations contained 0.5 per cent of the Harris yeast-vitamin powder. Increasing the concentration to 1.0 per cent gave the mothers with young 60 mg. additional of that yeast extract. It could hardly be argued that 60 mg. of the Osborne and Wakeman yeast extract prepared by the Harris Laboratories could add enough more of a mineral element or group of inorganic ions, or enough more of a nitrogenous substance or substances than that already contained in the rations to become the limiting factor in lactation. It is, of course, possible that yeast may contain several vitamins performing different physiological functions. In that case the Harris yeast-vitamin powder may carry a dietary complex which, in larger concentrations, is specifically beneficial

in lactation. Until it is shown, however, that there are present in yeast vitamins other than water-soluble B necessary for the growth and reproduction of mammals, it is most reasonable to assume at present that the water-soluble B vitamin is the factor involved.

The charts clearly indicate that litters of mothers partaking of rations containing ethereal extracts of the wheat embryo (Series II experiments) fortified with larger concentrations of the water-soluble B vitamin make significant gains from the beginning of lactation, as compared with little incipient gains of litters of mothers receiving the same rations but with lesser amounts of the yeast extract. This is further evidence of the prominent rôle played by the water-soluble B vitamin in lactation in the Series II experiments.

Effect of an Ethereal Extract of Hemp-Seed on Reproduction.

CHART V, LOT 468. Female 2001 had nine young and was given six, weighing 33 gm., to rear. On the 2nd day after birth the litter gained only 1 gm., and on the 3rd day only 2 more gm. The increase in the potent yeast extract at point "x" resulted in the successful weaning of five young, but the laxative effect of the hemp-seed oil, together with the gastrointestinal disturbances young animals undergo the 1st month and a half on a skimmed milk powder diet, when fed at a 50 per cent plane of intake, was more than the young could endure. The diarrhea brought them to such a miserable physical condition that it was decided to eliminate this group of young animals.

Female 1999 had six young which weighed 22 gm. On the 3rd day the mother reduced them to four which are being successfully reared.

Effect of Ethereal and Acetone Extracts of Yellow Corn on Reproduction.

CHARTS VI and VII, LOT 469. Ration 469 contained 3 per cent of an ethereal extract of yellow corn. After 70 days of growth the preparation of the ethereal extract was exhausted and was replaced by a 5 per cent acetone extract of yellow corn.

Female 2004 had the ethereal extract of yellow corn throughout her gestation period and was given the acetone extract on the day of the delivery of her litter. This mother had ten young, and was given six, weighing 30 gm., to rear. The effect of the increase of the water-soluble B vitamin concentration on lactation is indicated in Chart VI. The litter was successfully weaned and a healthy and vigorous second generation of this group is growing up.

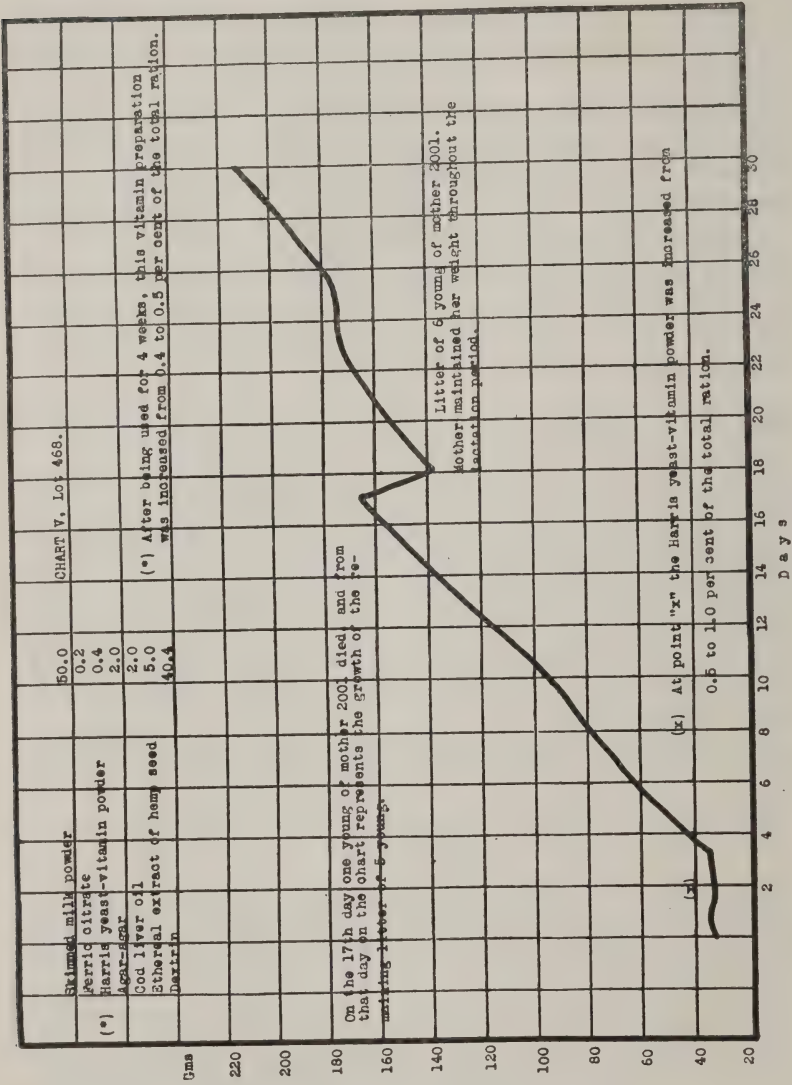


CHART V.

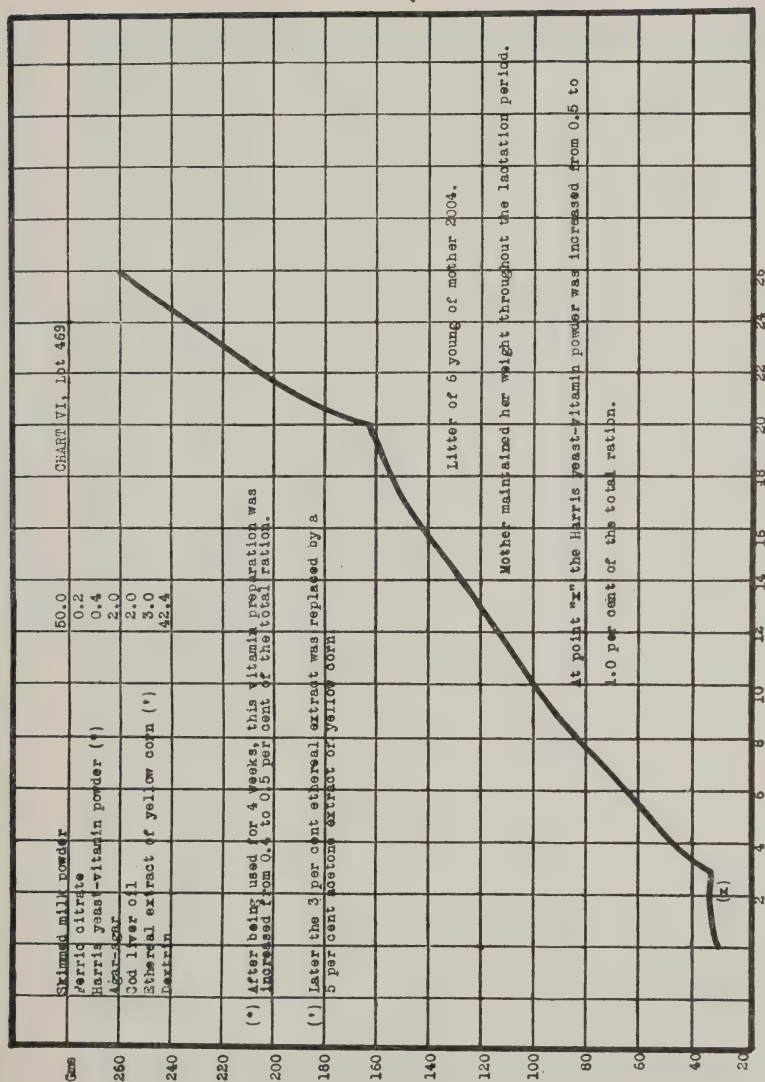


CHART VI.

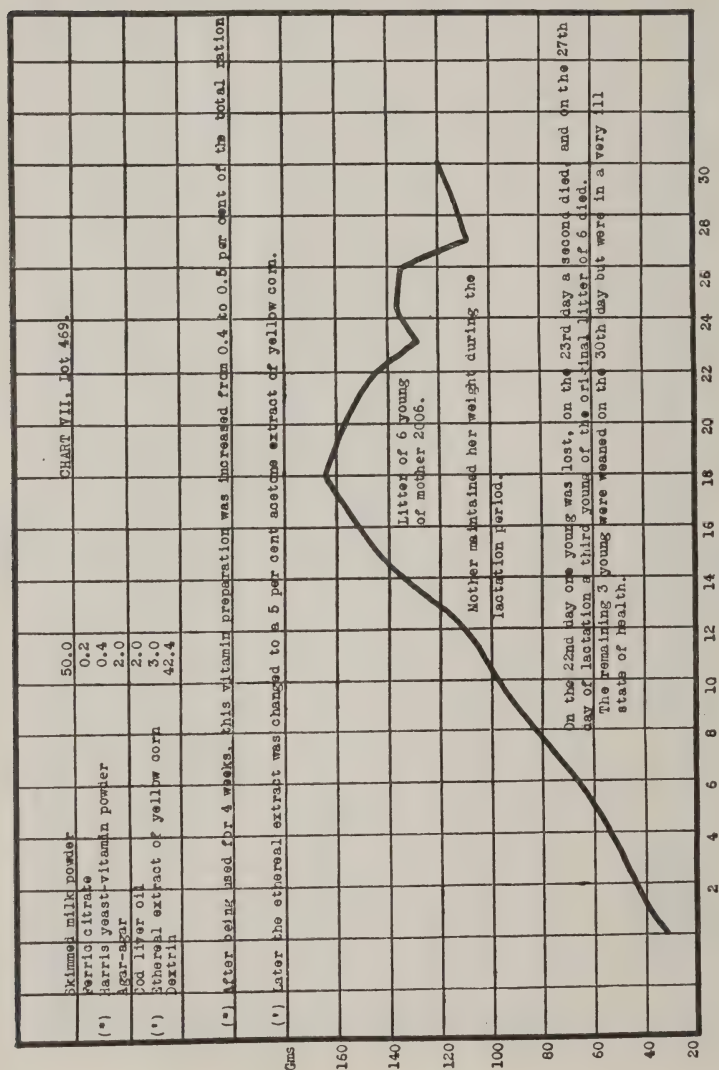


CHART VII.

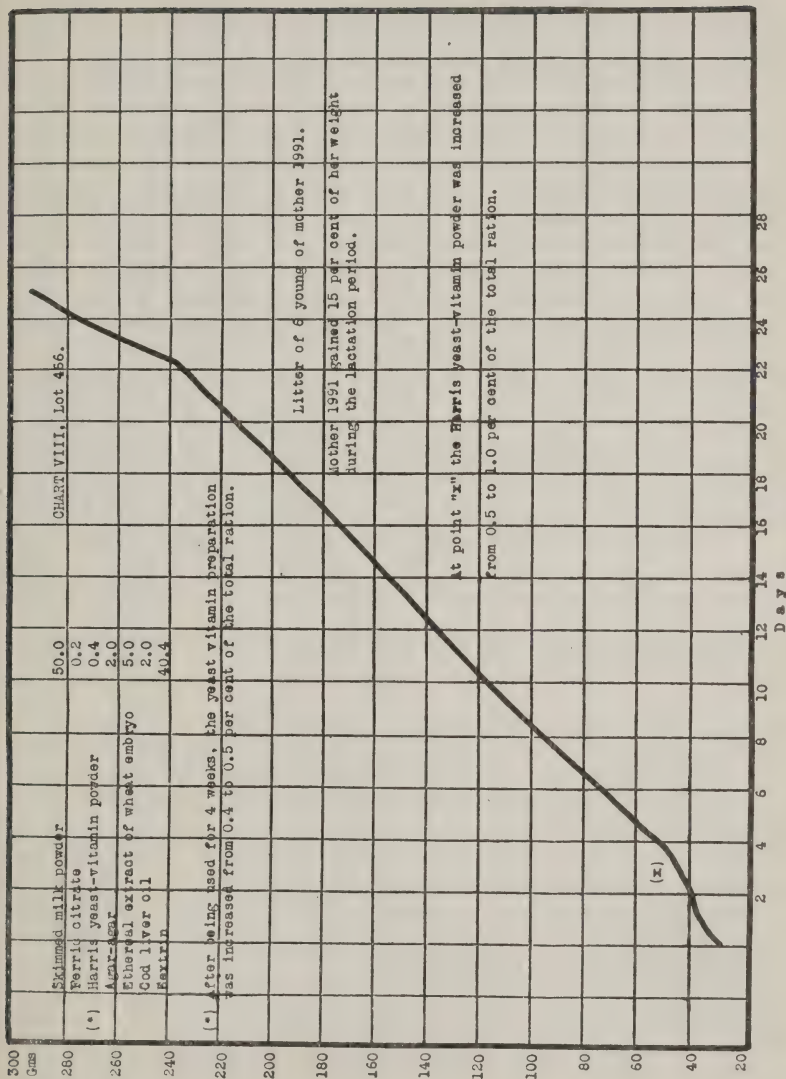


CHART VIII.

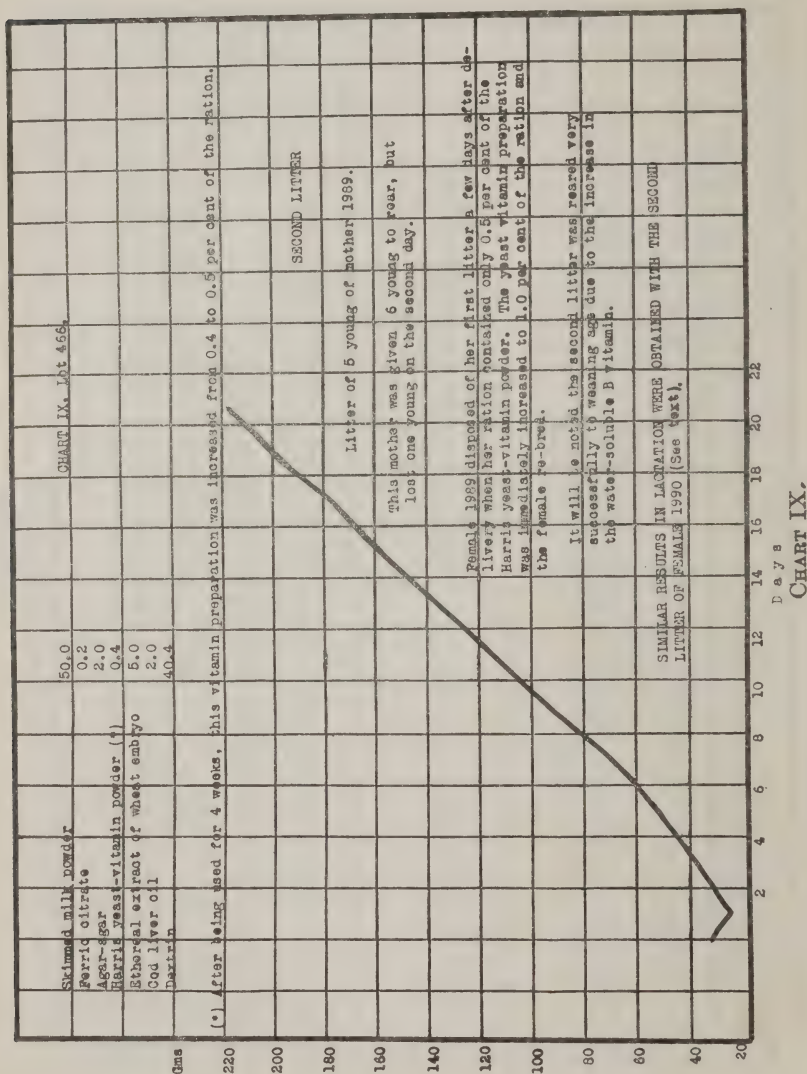


CHART IX.

Female 2006 had during the first half of her gestation period a 3 per cent ethereal extract and during the second half a 5 per cent acetone extract of yellow corn. This animal had nine young, was given six, weighing 30 gm., and reared her whole litter successfully for the first 18 days, but after the 18th day began to fail very badly in lactation. The only tentative explanation is that acetone may not extract the reproductive dietary complex from yellow corn as efficiently as ether. Female 2004 may have had more reserve of the ether-extracted complex stored up and this together with the acetone extractive had sufficient vitamin E to enable her successfully to nurse her young up to weaning age. The acetone extract of yellow corn had considerable solids, was thick in consistency, dark in color, and undoubtedly had extracted from the corn a different series of fatty acids from that found in the ethereal extracts, and possibly also some other lipoids. The ethereal extract was wholly in the liquid state and was lighter in color. More work, however, is necessary to settle completely the efficiency of acetone as compared with ether as a solvent of the reproductive vitamin from yellow corn. On the 30th day the three remaining young of Female 2006 weighed 39 gm. each, but were not in the very best state of health.

The third female, No. 2005, of this experiment had the acetone extract during the whole gestation period. She had seven young, was given six, reduced them to three on the 2nd day, to two on the 4th day, and disposed of the remaining two in the course of the next few days.

Effect of an Ethereal Extract of Wheat Embryo on Reproduction.

The greatest success in lactation was obtained with the ethereal extract of the wheat embryo.

CHARTS VIII and IX, Lot 466. This ration contained 5 per cent of an ethereal extract of wheat embryo and 0.5 per cent of the Harris yeast-vitamin powder. Females 1989 and 1990 had litters of nine and eleven young, respectively, and were given six young to rear, but the mothers disposed of their litters on the 2nd day. The third female, No. 1991, had ten young and was given six, weighing 27 gm., to rear. It will be noted by inspection of Chart VIII that almost as much growth was made by the litter between the 4th and 6th day as in the entire first 4 days of lactation. The response to the increase in the yeast extract is very suggestive in this case, but not conclusive. The outstanding fact, however, remains that this animal (Female 1991) reared her litter of six normally throughout the lactation period and a second generation of this group of animals is growing up successfully. The interesting fact is that the second generation is encountering considerably less diarrhea than their parents during the early part of their growth period.

Females 1989 and 1990 were rebred on the higher (1.0 per cent) concentration of the Harris yeast-vitamin extract. Female 1989, which failed with her first litter on the 0.5 per cent yeast extract-containing diet, had

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fifteen young with the second litter. She was given six, weighing 29 gm., to rear and successfully reared five young throughout the lactation period. A healthy second generation of this group is growing up. Female 1990 gave birth to twelve young (second litter), was given six, weighing 29 gm., to rear and successfully reared five young throughout the lactation period. This mother had failed with her first litter (see Chart IX).

The above evidence seems to corroborate the theory that the water-soluble B vitamin plays a hitherto unsuspected tremendous rôle in lactation, which in the Series II skimmed milk powder experiments resolves itself into a determining limiting factor in rearing of young.

CHARTS X and XI are self-explanatory. Two healthy, vigorous second generations are growing up on Ration 467 which contained 3 per cent of an ethereal extract of wheat embryo.

CHARTS XII and XIII, Lot 484. This ration contained 35 per cent of skimmed milk powder fortified with casein and gelatin, thus varying the quality of the amino acid content of the diet. No fertility was obtained on the control ration, No. 483, from which the wheat germ oil was absent. During the middle of the gestation periods of Females 2077 and 2079 (Lot 484) when it became apparent that several mothers were failing on skimmed milk powder rations containing ethereal extracts of wheat embryo, a strenuous attempt was made to improve the quality of Ration 484, as indicated in Charts XII and XIII. When this radical change was made in Ration 484 a similar change was made in the control ration, No. 483, with the exception that the wheat germ oil was left out. Females 2077 and 2079 reared their litters of five and six, respectively, throughout their lactation period very successfully, and healthy second generations of both of these groups of young animals are growing up. The interesting fact about this experiment is that an 80 per cent extract of ether-extracted wheat embryo in the concentration employed is able to replace 0.5 per cent of the potent yeast extract for lactation, even when the plane of skimmed milk powder intake is reduced from 50 to 35 per cent as a source of the water-soluble vitamin.

Female 2078 gave birth to only three young which were disposed of by the mother in a few days, for reasons not quite apparent at this time. The mammary gland of the mother was found dry. Possibly this individual female needed a larger concentration of the water-soluble B vitamin for lactation.

Lot 471. This lot received 5 per cent of commercial olive oil in the ration. Two females out of three gave birth to litters that were very pale and emaciated looking and were disposed of by the mothers in a few days. The mammary glands of the mothers were dry. It seems that commercial olive oil contains enough of the reproductive dietary complex

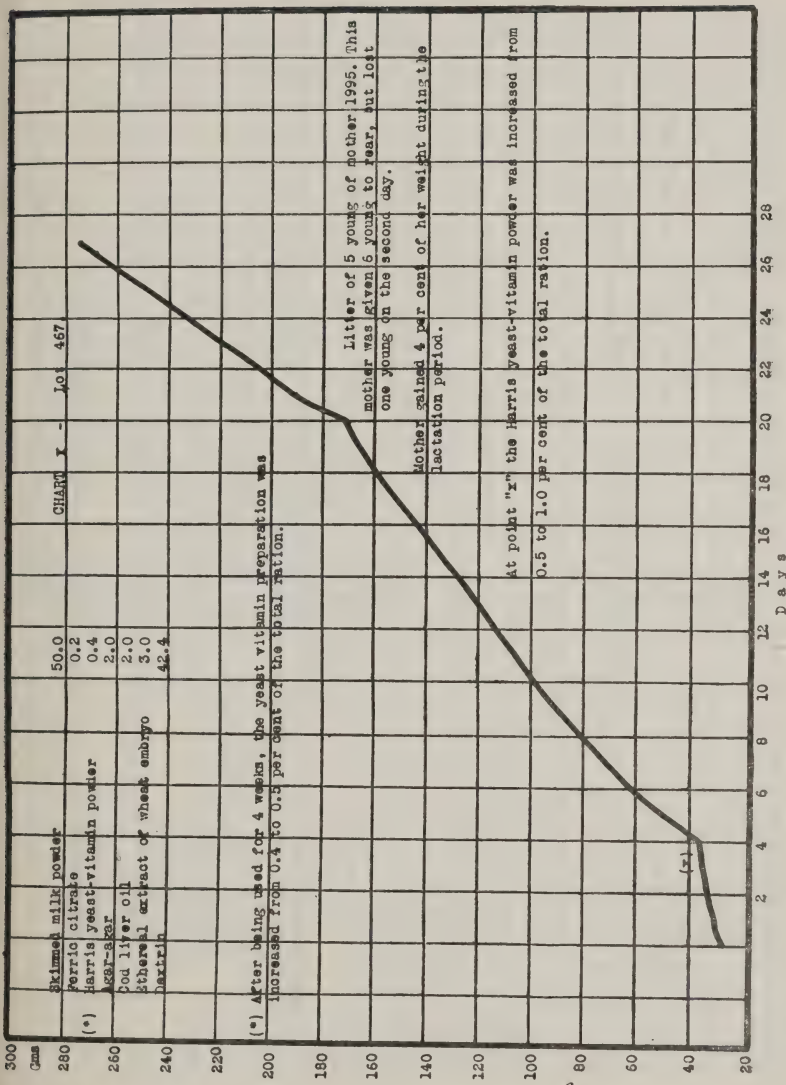
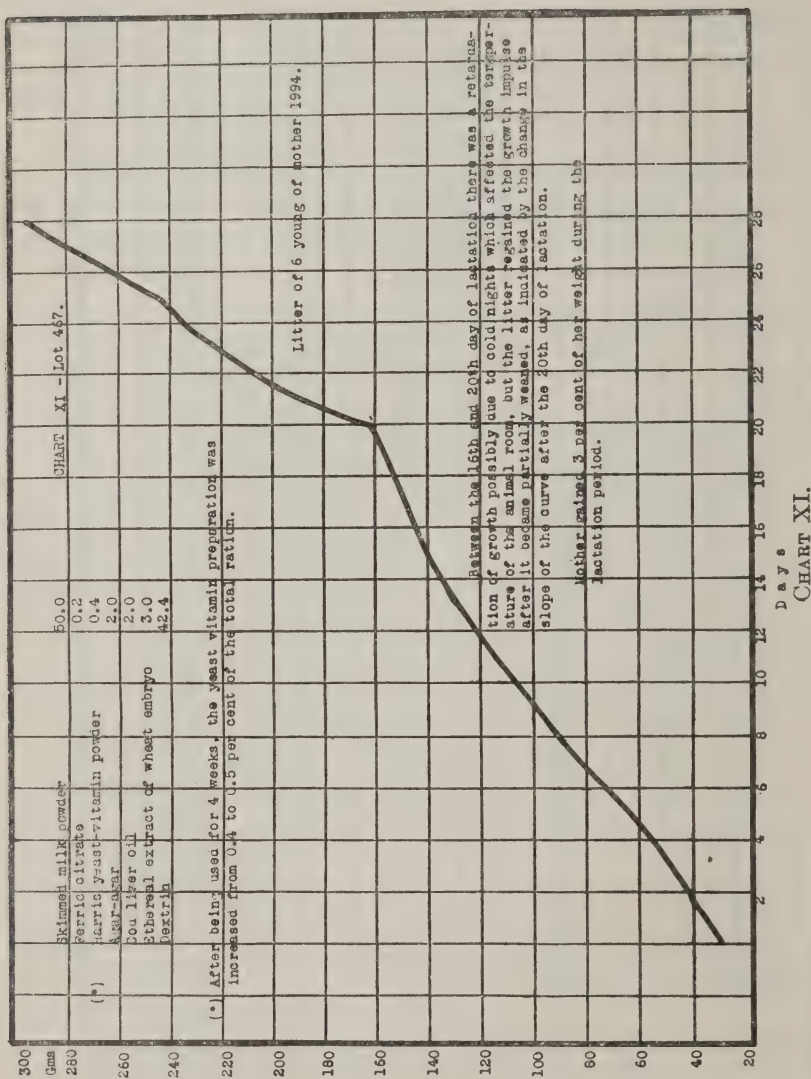
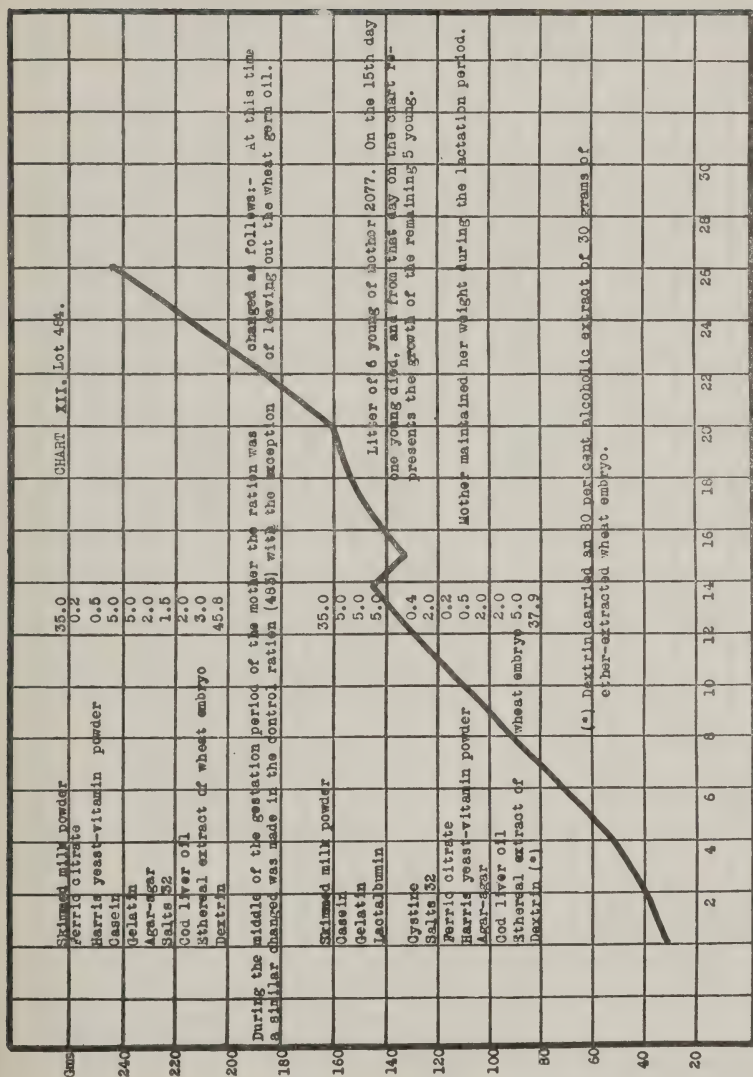
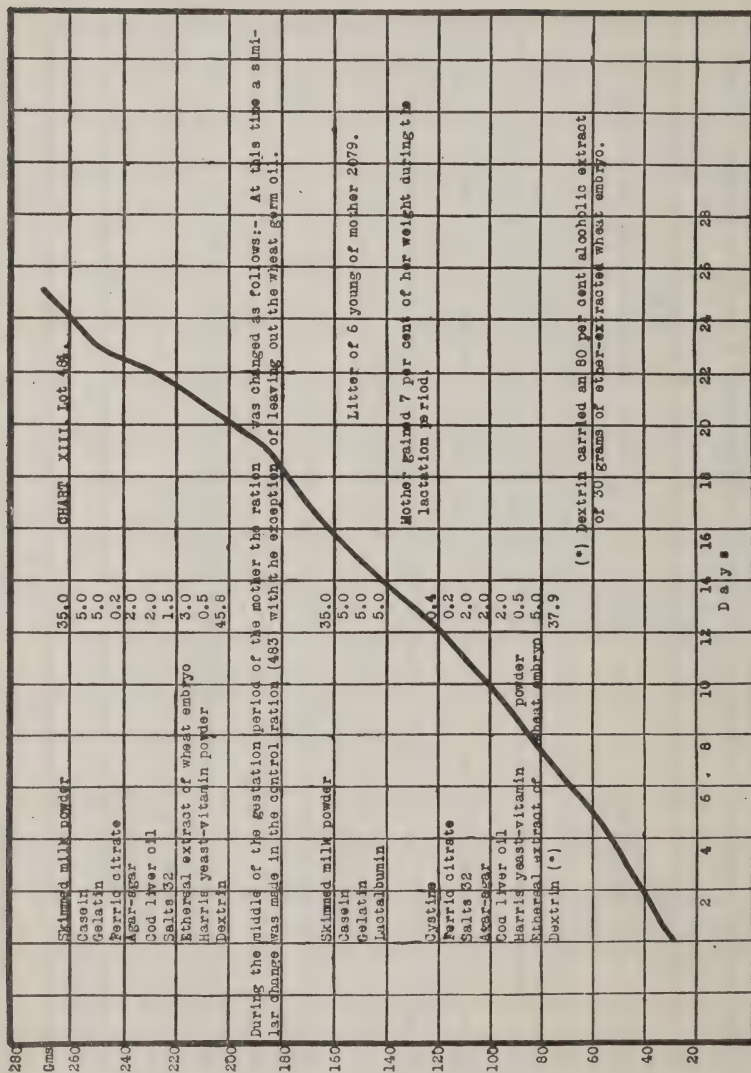


CHART X.





Days
CHART XII.



for fertility, but not enough for proper nourishment of the fetus during gestation, since the young were born in abnormal condition.

No fertility was obtained with commercial linseed, coconut, or sesame oils when introduced to the extent of 5 per cent in the ration.

CONCLUSION.

The original hypothesis of the author formulated in the early part of 1919 that the total milk proteins, as found in skimmed milk powder, although excellent for growth, are inadequate for reproduction, must be abandoned because of the nature of the experimental findings reported in this paper. Skimmed milk powder, when incorporated to the extent of 50 per cent in a ration, thus introducing 17.5 per cent protein, furnishes an excellent quality and sufficient quantity of amino acids for the whole cycle of reproduction, which includes ovulation rhythm, placental function, fertility of healthy normal litters born after the complete gestation period, and successful lactation. When fortified with 0.2 per cent ferric citrate, skimmed milk powder, fed at a 50 per cent plane of intake, furnishes sufficient mineral elements of excellent quality for reproduction. After the anti-xerophthalmic and antirachitic vitamins in the form of 2 per cent cod liver oil, and roughage by the addition of 2 per cent agar-agar, have been supplied, two dietary factors, however, for successful reproduction are still deficient in skimmed milk powder: (1) A hitherto unrecognized organic factor, vitamin E, found in ethereal extracts of wheat embryo, yellow corn, and hemp-seed; and (2) a high concentration of the water-soluble B vitamin, a concentration much greater than that required for growth.

Five young were successfully weaned on a ration which contained a 3 per cent ethereal extract of yellow corn, which was later changed to a 5 per cent acetone extract of yellow corn. A healthy second generation of this group is growing up.

Five young were successfully weaned on a ration containing an ethereal extract of hemp-seed, but owing to the laxative effect of the hemp-seed oil and to the gastrointestinal disturbances young undergo the first 5 to 7 weeks on skimmed milk powder diets, it was not possible even to attempt to rear a second generation of this group of animals.

The most successful results were secured with an ethereal extract of wheat embryo as a source of vitamin E. Healthy

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litters were born to females partaking of rations containing 5 and 3 per cent of wheat germ oil. Some of the litters would have died early during lactation, but were saved by increasing the water-soluble B vitamin concentration of the diets of their mothers at the critical period. With the higher concentration of the water-soluble B vitamin, young start out with excellent initial growth. *Six healthy, vigorous second generations are growing up on the wheat germ oil experiments, while twelve females on control experiments, with no vegetable oils present, did not even become pregnant.*

SUMMARY.

1. Positive evidence is presented showing that the ethereal extracts of yellow corn, wheat embryo, and hemp-seed contain an organic factor, designated as vitamin E, which is essential for reproduction.

2. Fertility was also secured with commercial cottonseed oil and commercial olive oil, when introduced to the extent of 5 per cent of the ration, but not with commercial cocoanut, linseed, or sesame oils.

3. The requirement of the water-soluble B vitamin for normal mammary gland function is much greater than that for growth.

4. Skimmed milk powder, when introduced to the extent of 50 per cent in the ration, furnishes amino acids sufficient in quantity and of excellent quality for reproduction.

5. Skimmed milk powder, fortified with 0.2 per cent ferric citrate, fed at a 50 per cent plane of intake, furnishes sufficient mineral elements of excellent quality for reproduction.

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THE NATURE OF THE SUGAR IN NORMAL URINE.

I. A COMPARISON OF THE GLUCOSE EQUIVALENT OF VARIOUS SUGARS IN DIFFERENT METHODS FOR THE DETERMINATION OF GLUCOSE.

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In an investigation concerning the nature of the sugar in normal urine, it was believed that some information might be obtained by a comparison of the results obtained by various methods with those obtained by the same methods upon various pure sugars. The methods originally employed upon the urines were the Folin-Berglund (1), Shaffer-Hartmann (2), Benedict and Osterberg's sodium carbonate-picrate (3), and the same authors' sodium hydroxide-picrate-acetone method (4). Later, when the concentrated, partly purified sugars were to be examined, it was decided to employ the MacLean (5) and the Sumner (6) methods also. In this paper, we report the results obtained upon the pure sugars by the various methods.

The figures in Table I represent the glucose equivalent of 1 mg. of the particular sugar; that is, the amount of glucose indicated by the particular method for each milligram of sugar. The amounts of sugar were so chosen as to bring the depth of color or the amount of titrated material within the prescribed range. In every case in which the matter was investigated, the glucose equivalent varied with the concentration of the sugar. Therefore, the values in the table are only approximately correct. They should not be used over all ranges of concentration if accurate results are desired.

The results obtained upon various sugars by the use of the sodium carbonate-picrate method of Benedict and Osterberg have recently been reported by others (7, 8). In general, the values

agree fairly well with those reported in this paper. However, our values for the glucose equivalent of galactose and mannose are higher than those reported by Willaman and Davison, who found them to be 0.883 and 1.00, respectively. Our value for fructose is slightly lower than that reported by either Thomas and Dutcher (8) or Willaman and Davison (7). Our value for the glucose equivalent of maltose (0.82) is intermediate between that reported by Thomas and Dutcher (0.91) and that reported by Willaman and Davison. Possibly the variations in the concentrations employed may account for the differences in the factors obtained.

TABLE I.

Comparison of the Reducing Action of Various Sugars with That of Glucose.

Sugar.	Glucose equivalents of 1 mg. of sugar.					
	Methods.					
	Folin-Wu.	Shaffer-Hartmann.	MacLean.	Benedict and Osterberg.		Sumner.
				Na ₂ CO ₃	NaOH	
	mg.	mg.	mg.	mg.	mg.	mg.
Xylose.....	0.94	0.96	0.83	1.09	1.28	1.14
Arabinose.....	0.80	0.80	0.80	1.02	1.17	1.17
Fructose.....	0.91	0.90	0.94	0.96	1.08	1.00
Galactose.....	0.75	0.80	0.77	0.95	1.04	0.95
Mannose.....	0.58	0.81	0.93	1.08	0.86	0.76
Maltose.....	0.40	0.42	0.46	0.82	0.77	0.70
Lactose.....	0.45	0.53	0.51	0.78	0.74	0.70
Glucosamine.....	0.92	0.90	0.95	0.98	0.54	0.34

It is interesting to observe that, except in the case of mannose, the copper methods give values that agree fairly well among themselves and that the picrate and dinitrosalicylate methods also agree with each other, except when employed upon mannose and glucosamine. The difference between the two groups of methods is striking. The copper methods, almost uniformly, give lower values than do the methods of the second group. This is particularly striking with the disaccharides, maltose and lactose.

With glucosamine, the copper methods and the sodium carbonate-picrate method give nearly identical values, but the sodium

hydroxide-picrate method gives a lower figure and the dinitro-salicylate method a still lower. Similar, but less marked, differences are found in the values obtained by the methods of the second group upon mannose. It is interesting to note that the picrate-acetone method depends upon the reduction, not of sodium picrate, but of the product of the reaction between sodium picrate, acetone, and sodium hydroxide. This was shown to be the case by Benedict and Osterberg (4), and has been confirmed in this laboratory by another method. In mixtures such as are used in the determination of sugar, the acetone immediately acts upon the sodium picrate so that no precipitate is produced upon reacidification and treatment with nitron.

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THE NATURE OF THE SUGAR IN NORMAL URINE.

II. THE SUGAR EXCRETION UPON VARIOUS DIETS AND THE INFLUENCE OF DIET UPON GLUCOSE TOLERANCE WITH SOME REMARKS ON THE NATURE OF THE ACTION OF INSULIN.

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INTRODUCTION.

That normal human urine probably contains one or more carbohydrates has been generally believed for a long time, but there has been no agreement as to the nature of this carbohydrate. A review of the older literature is given by Schulz (1).

The fact that a part of the reducing action may be lost upon treatment with yeast has generally been accepted as evidence of the presence of glucose. Several observers have also obtained an osazone melting at temperatures between 190° and 200°. Since glucosazone, galactosazone, lactosazone, and maltosazone all melt at about this temperature, the melting point alone cannot be considered as identifying the osazone.

The question as to the nature of the sugar has recently been revived by Benedict, Osterberg, and Neuwirth (2) and by Folin and Berglund(3). The latter give a complete summary and discussion of the more recent literature. The former regard glucose as a normal constituent of the urine whereas the latter deny that it is. That not all the sugar of the urine in glucose is recognized by Benedict and his associates, but they believe that about one-third is. This view is based chiefly upon the fact that about one-third of the reducing action of the urine upon alkaline picrate solutions disappeared after incubation of the urine with yeast and added glucose. This fraction of the total urinary sugar was increased by the ingestion of glucose.

It is quite true that glucose is one of the few sugars that are fermented by yeast, if we mean by that statement that it is one of the few that will yield alcohol and carbon dioxide when incubated with yeast. But that gives lit-

* A preliminary report was read before the American Society of Biological Chemists at Toronto, December, 1922.

the reason for assuming that a diminution of the reducing action of a urine, containing at most 0.1 per cent of sugar, upon treatment with yeast and other organisms, is due to the fermentation of glucose. It is true that Gilbert and Baudouin (4) observed a similar loss of reducing action upon the treatment of boiled and filtered urine with a pure culture of yeast, but no one has ever demonstrated the production of either alcohol or carbon dioxide. That yeast may utilize carbohydrates of other than the usually fermented types has been abundantly demonstrated. Bokorny (5), Cross, Bevan, and Smith (6), and Cross and Tollens (7) have all shown that pentoses may be utilized by yeast, without the production of alcohol. In our experiments, mixtures of urine and yeast kept for 24 hours at room temperature or at 37° sometimes lost in reducing action, sometimes gained, and sometimes were quite unaffected.

Benedict and Osterberg (8) have recently severely criticized the failure of Folin and Berglund (3) to consider as significant the small increases in urinary sugar observed after the ingestion of glucose. But these slight increases, even if real and not due to errors in the collection and measurement of such small volumes of urine or to errors in the determination of sugar, amount to, at most, 0.03 per cent of the amount of glucose ingested. The presence, as an impurity, of this quantity of a non-assimilable sugar in the glucose used would be quite beyond detection by our ordinary methods of analysis.

In the same paper, Benedict and Osterberg report the results of their own experiments upon the effect of subcutaneous injections of glucose upon the output of urinary sugar. This was uniformly increased. But we are quite unable to understand why these small increases in the amount of sugar in the urine observed after the injection of a very large amount of glucose should at all indicate that urinary sugar is normally derived from metabolic glucose. Such amounts are not normally absorbed in anything like so short a time, and the condition of the glucose in the blood, after the administration of such large amounts of glucose by this unnatural channel, may be quite different from the normal. That may well be the explanation of Benedict and Osterberg's finding of 1.2 per cent of sugar in the urine with only 0.12 per cent in the blood. Benedict and Osterberg themselves realized this (8), but seemed unable to draw from it what seems to us to be the inevitable conclusion, which is, that the results of their experiments indicate that glucose is not, normally, present in the urine of dogs.

Furthermore, examination of their figures discloses that, except in one experiment (Table I, No. 3), the amount of extra sugar in the urine following the administration of glucose was between 0.3 and 0.7 per cent, generally between 0.4 and 0.5 per cent, of the amount injected, although that ranged from 0.4 to 8 gm. per kilo of body weight. In the one exceptional experiment, the additional amount excreted was almost 1.5 per cent of that injected, although this was only 0.4 gm. per kilo. In all the experiments, the glucose was administered in 60 per cent solution, sterilized by boiling. This suggests the possibility that a small part of the glucose had become polymerized or changed to a non-assimilable form before injection. In-

deed, Benedict and Osterberg noted that the increase in the excretion in the urine was not only in the amount of fermentable sugar but in that of non-fermentable and in that of hydrolyzable sugar also. They believed this hydrolyzable sugar to be an intermediate in the formation of glycogen. If this were so, the amount that escaped when the glycogenic functions of the body were most severely taxed should have constituted a greater fraction of the amount ingested than when the strain on the glycogen-forming organs was not so great. But this was not the case. Moreover, the delayed excretion of a product normally intermediate between glucose and glycogen is quite inexplicable. After the strain on the glycogenic functions was over, the intermediate products should have been converted into glycogen and not excreted into the urine. But, if this hydrolyzable sugar represents a polymer present in the material injected, the delayed excretion is quite to be expected. It is very frequently observed after the injection of foreign substances.

The results of Felsher and Woodyatt (9) are of interest in this connection. These observers found that in dogs receiving intravenous injections of glucose at fixed rates, there was only a very slight increase in the excretion of sugar in the urine up to a certain critical rate of glucose injection and concentration in the blood, after which it increased very rapidly. Examination of their figures again shows that this slightly increased excretion in the urine which occurred with the lower rates of injection varied only between 0.2 and 0.7 per cent, and usually between 0.3 and 0.4 per cent, of the amount injected, although this varied from 0.3 to 1.3 gm. per kilo per hour. Felsher and Woodyatt sterilized their glucose in 20 per cent solution in an autoclave. The temperature and duration are not stated in the paper, but in a private communication from Dr. Woodyatt we are informed that the solutions were heated to 120°, kept at this temperature 20 minutes, and then allowed to cool. It seems to us quite possible that some of the glucose was polymerized or changed in this process. The possibility that the glucose injected was not quite pure was mentioned by Felsher and Woodyatt as an explanation of the results obtained.

That glucose solutions are changed when heated above 100° was noted as long ago as 1885. Degener (10) reported that glucose solutions heated to 130° decreased in reducing action upon alkaline copper solutions but increased in rotating power. This was confirmed by von Lippman who found that this change occurred even at 100° (11). In this laboratory, we have found that a 38 per cent solution of glucose, which in a 1 dm. tube showed the calculated rotation of 20.4°, after heating to 100° for 7 hours, had a rotation of only 19.4°; and after heating to 120° for 7 hours had a rotation of 19.8°. The first change upon heating may result in a decrease in rotation, with a subsequent increase, or the temperature may decide which of the many possible reactions shall occur. The original solution was colorless; the heated solutions were both yellow. The reaction of the original solution was nearly neutral, pH 6.0; the heated solutions were distinctly acid, pH 4.3. However, the total amount of acid formed was slight, 25 cc. requiring only 0.25 cc. of 0.1 N NaOH, using phenolphthalein as indicator.

As has already been stated Folin and Berglund do not believe that the urinary sugar is glucose or bears any relation to normal glucose metabolism. From the results of their determinations of the concentration of sugar in the blood and urine of normal persons before and after the ingestion of glucose and other sugars, they conclude that the sugar of the urine represents a mixture of difficultly assimilable sugars present in the foodstuffs, of carbohydrates altered in cooking, and of some sugar derived from an endogenous source.

If it could be definitely established that glucose is not present in human urine under anything like normal conditions, the development of a delicate, specific reaction for glucose should be of considerable value in the detection of diabetes. If, however, glucose is normally present in the urine, the development of a technique for the detection of early or slight deficiencies in carbohydrate tolerance must take a decidedly different course. The nature of the sugar excreted in the urine of men and dogs upon a carbohydrate-free diet, most, or all, of which both Benedict and Folin recognized as not being glucose, also appeared to deserve investigation. It might be derived from nucleic acids, inosinic acid, etc., and might offer a better means of measuring nucleic metabolism than any other at present available.

EXPERIMENTAL.

The experiments to be reported in this paper were intended to be part of a more extensive investigation. It was intended to isolate, or at least to concentrate, the sugars from urine and to study their properties. Various circumstances have interfered with the completion of that part of the work and it has been thought best to report the results of the metabolism experiments. An unexpected effect of previous diet upon glucose tolerance was obtained. This has also been observed by others.

Four different methods were used for the determination of urinary sugar. Two of these (Benedict and Osterberg (12, 13)) were picrate and two (Folin and Berglund (14) and Shaffer and Hartmann (15)) were copper reduction methods. In one method of each group, the determination is carried out in the presence of most of the nitrogenous constituents of the urine. In the others, the determinations are made upon the filtrate obtained after treating the urine with mercuric nitrate and sodium bicarbonate.

Our technique for this precipitation was somewhat different from that employed by others. In order to avoid undue dilution of the urine and waste of mercury, measured quantities of a saturated solution of mercuric nitrate (prepared by adding mercuric oxide to a boiling mixture of 1 volume of concentrated nitric acid and 2 volumes of water until no more dissolved and then allowing to cool) were added to the urine until a small amount of sodium bicarbonate dropped into the mixture was surrounded, not by a white, but by a red or brown, precipitate. Sodium bicarbonate was then added until the mixture was no longer acid to litmus (it became alkaline during the filtration) and the filtrate was freed of mercury with H_2S and of the latter by aeration.

Inorganic phosphate was determined by the method of Bell and Doisy (16) or by Briggs' modification thereof (17). The Folin-Wu method (18) was used for the determination of uric acid. All determinations were made in duplicate and, whenever anomalous results were obtained, the determinations were repeated, even though duplicates had agreed. Such results were usually confirmed.

Sugar Excretion and Sugar Tolerance in Man upon Various Diets.

The subjects of the experiment were one man and two dogs. The results of the experiments upon the human subject, I. G., male, age 35 when the experiments were begun, weight 71 kilos, are summarized in Tables I to IV and VI. At first, the diet consisted chiefly of carbohydrates and included considerable quantities of fruit, toast, caramels, etc., in order to include the maximal, normal amount of foreign non-assimilable carbohydrates, which, according to Folin and Berglund, give rise to the greater part of the urinary sugar. The food supplied fully 3,000 calories. The amounts of sugar excreted in the urine were quite constant, except for 1 day, on which an unusual amount of honey and syrup was consumed, particularly at one meal. Except for this 1 day, the amounts are far smaller than those reported by Benedict, Osterberg, and Neuwirth for their subjects, particularly when the difference in body weight is considered.

The diet was now changed to one as nearly free from carbohydrate as could well be obtained (Table II). The subject ate heartily and the energy intake was at least 3,000 calories per

TABLE I.
Excretion of Sugar on a Carbohydrate-Rich Diet.

Subject I. G.		Urine.					Diet.			
		Date.	Volume. cc.	Nitrogen. gm.	Sugar.			Breakfast.	Lunch.	Dinner.
					Benedict NaOH. gm.	Folin- Berglund. gm.	Benedict Na ₂ CO ₃ . gm.			
1922	Oct. 7	1,160	10.89	0.931	0.602	0.654	0.379	2 bananas, puffed rice, cream, milk, roll, honey.	Baked potatoes, carrots, peas, bread, butter.	Baked beans, celery, bread, butter, candy.
	"	750	10.10	0.802	0.528	0.708	0.385	Banana, peach, puffed rice, cream, milk, roll, butter.	Bread, Swiss cheese, chocolate.	Tomato, green pepper, lettuce, bread, butter, grapes, grape juice, ice cream.
	"	550	12.10	0.710	0.542	0.738	0.458	Baked apple, puffed rice, cream, milk, roll, but- ter.	Waffles, honey, rice pud- ding, roll, butter.	Noodles with sugar and cinnamon, potato, to- mato, lettuce, baked apple.
	"	720	10.33	0.655	0.479	0.686	0.426	Baked apple, puffed rice, cream, milk, roll, but- ter.	Salad of egg, asparagus, tomato, lettuce, cel- ery, potato. Stewed prunes.	Chicken, rice, apple sauce, candy.
	"	1,300	9.71	0.924	0.761	0.687	0.542	Baked apple, grape nuts, cream, milk, roll, grape jelly.	Toast, orange marmalade, 2 cups tea with cream and sugar, pine- apple pie.	Potatoes, lettuce, rice, blackberry jelly, 3 large caramels.

Oct. 12	1,200	9.28	0.843	0.598	0.754	0.495	Peaches, cream, grape nuts, milk, roll, honey.	Toast, orange marmalade, peach pie, ice cream.	Potatoes, string beans, apple sauce, toast, 3 large caramels.
" 13	1,720	9.00	1.110	0.842	1.123	0.670	Peaches, cream, grape nuts, milk, roll.	Waffles, honey, canned pineapple. 1½ hrs. later, 2 large pears.	Potatoes, apple sauce, nuts, dates, apple pudding, 3 large caramels.
" 14	960	8.71	0.808	0.551	0.731	0.508	Prunes, dates, grape nuts, milk.	Vegetable soup, stuffed dumplings, ice cream.	Baked beans, bread, butter, 4 bananas, 3 large caramels.

day. On the 3rd day, the nitrogen excretion in the urine was 34.8 gm., but it dropped the next day to 29.4 gm., after which it was quite constant. It was impossible to maintain the initial high level of protein intake. The amount of sugar excreted on

TABLE II.

Excretion of Sugar on a Carbohydrate-Free Diet and Influence of Added Carbohydrate.

Date.	Urine.							
	Volume.	Nitrogen.	Sugar.					
			Benedict NaOH.	Folin- Berglund.	Benedict Na ₂ CO ₃ .	Shaffer- Hartmann.	Creatinine.	Creatine.
1922	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Oct. 18.								
7 a.m.-11.30 p.m.....	1,120	23.25	0.497	0.353	0.418	0.218	1.560	0.256
11.30 p.m.-7 a.m.....	560	11.58	0.245	0.176	0.165	0.110	0.739	0.033
Total.....	1,680	34.83	0.742	0.529	0.583	0.328	2.299	0.289
Oct. 19.								
7 a.m.-11.30 p.m.....	920	19.86	0.423	0.300	0.365	0.222	1.447	0.208
11.30 p.m.-7 a.m.....	410	9.58	0.189	0.121	0.145	0.097	0.568	0.044
Total.....	1,330	29.44	0.612	0.421	0.510	0.319	2.015	0.252
Oct. 20.*								
7 a.m.-11.30 p.m.....	820	19.22	4.410	4.320	4.570	4.340	1.700	0.054
11.30 p.m.-7 a.m.....	375	8.91	0.250	0.207	0.151	0.074	0.686	0.064
Total.....	1,195	28.13	4.660	4.537	4.720	4.414	2.386	0.118
Oct. 21.†								
7 a.m.-11 p.m.....	820	17.96	1.155	0.870	1.033	0.540	1.695	0.100
11 p.m.-7 a.m.....	400	8.55	0.308	0.204	0.243	0.142	0.698	0.027
Total.....	1,220	26.51	1.463	1.074	1.276	0.682	2.393	0.127

* 71 gm. of glucose with each of three meals.

† 100 gm. of grape nuts taken with each of three meals.

this diet was almost as great as when the ingestion of foreign sugar was at its height. On the 5th day of this carbohydrate-free diet, 71 gm. of glucose, or 1 gm. per kilo of body weight, were taken with each of the three meals. The sugar excretion rose to over

4 gm. and measurement of the volume of CO_2 produced upon treatment with yeast indicated that 3.4 gm. were glucose. The analysis of the night urine showed that the effect of the glucose ingestion was transient. On the next day, 100 gm. of grape nuts were taken with each meal. This was selected as an example of a food in which cooking had produced the maximal change in the nature of the carbohydrate.¹ There was a rise in the excretion of sugar, but this was not so great as on the previous day and again the excretion returned to the normal before morning.

On the 3rd and 4th day of this experiment (1st and 2nd days of the table), the reaction with sodium nitroprusside showed that there was a small amount of acetone in the urine, but this was not sufficient to affect the determinations of creatine and creatinine, which were made by Folin's original methods. That the intake of food was quite constant is shown by the figures for the nitrogen and creatinine excretion. There was a slight creatinuria which was abolished by the addition of glucose to the food. The ingestion of 300 gm. of grape nuts, following so closely the addition of 213 gm. of glucose, made it necessary to reduce the intake of other foods. It was simply impossible to eat more.

The excretion of 3.4 gm. of glucose after the ingestion of three times 71 gm. of glucose was very surprising for the subject had repeatedly taken 100 and even 200 gm. of glucose after a night's fast with apparently perfect tolerance. The experiment was therefore repeated upon another diet. Purely as a matter of convenience, this contained 800 cc. of milk per day. For the rest, it consisted chiefly of potatoes, crackers, bread, and a small quantity of butter. It was the same each day. The excretion of sugar was considerable. The 1st day shown in Table I, I was

¹ Grape nuts are, essentially, the well toasted crumbs of a bread, in the baking of which the starch has been exposed to the action of malt. The analysis supplied us by the manufacturer is: protein, 12 per cent; maltose, 17 per cent; carbohydrates other than fiber, 65.3 per cent. According to our own analyses, 38 per cent are soluble carbohydrates, the reducing action of which is equivalent to that of 16 per cent maltose by the Folin-Wu, Shaffer-Hartmann, and Benedict-Osterberg sodium hydroxide-picric-acetone methods and to that of 23 per cent maltose by Benedict and Osterberg's sodium carbonate-picric method. After fermentation, these values became, approximately, 6 and 9 per cent, respectively. The iodine reaction indicated the presence of a small amount of dextrin.

the 3rd of the experiment. On the 5th day, 71 gm. of glucose were taken with each meal. The total amount of sugar in the urine was not only not increased but was rather diminished. The reduction was much less marked in the values obtained by the

TABLE III.

Excretion of Sugar on a Constant Carbohydrate-Rich, Fat-Poor Diet Containing 800 Cc. of Milk. Influence of Added Glucose.

Date.	Urine.					
	Volume.	Nitrogen.	Sugar.			
			Benedict NaOH.	Folin- Berglund.	Benedict Na ₂ CO ₃ .	Shaffer- Hartmann.
1922	cc.	gm.	gm.	gm.	gm.	gm.
Nov. 1.						
7 a.m.-11.15 p.m.....	820	8.15	1.277	0.752	1.230	0.820
11.15 p.m.-7.15 a.m.....	360	3.39	0.284	0.170	0.251	0.167
Total.....	1,180	11.54	1.561	0.922	1.481	0.987
Nov. 2.						
7.15 a.m.-11.15 p.m.....	820	7.62	1.581	1.155	1.695	1.225
11.15 p.m.-7.15 a.m.....	350	3.26	0.275	0.179	0.284	0.171
Total.....	1,170	10.88	1.856	1.334	1.979	1.396
Nov. 3.*						
7.15 a.m.-11.30 p.m.....	1,000	7.65	1.285	0.714	1.210	0.719
11.30 p.m.-7.30 a.m.....	520	3.06	0.346	0.157	0.289	0.156
Total.....	1,520	10.71	1.631	0.871	1.499	0.875
Nov. 4.						
7.30 a.m.-11.15 p.m.....	1,120	7.26	1.212	0.749	1.232	0.794
11.15 p.m.-7.15 a.m.†.....	180	2.14	0.193	0.120	0.193	0.128
Total.....	1,300†	9.40	1.405	0.869	1.425	0.922

Diet.

Breakfast: Baked apple, shredded wheat biscuit with milk, cocoa made with milk and sugar, roll and butter.

Lunch: 150 gm. of Graham crackers, 500 cc. of milk.

Dinner: 57 gm. of sausage, boiled potatoes, pickled beets, stewed pears and quinces, cake, chocolate, 30 gm. of bread.

* 71 gm. of glucose added to each meal.

† Some urine lost.

picrate methods than in those obtained with the copper methods. It would seem as if there had been a marked diminution in the excretion of some substance that reacts much more strongly with copper solutions than with picrate solutions. But no such substance is known. The same effect would have been produced by a diminution in the excretion of one substance and a simultaneous increase in the excretion of another that reacts more markedly with picrate solutions than with copper solutions. In the preceding paper it has been shown that all the sugars investigated, particularly the disaccharides, have higher glucose equivalents with the picrate methods than with the copper methods. It seems quite possible that the rather large sugar excretion on the control days of this period was due, in part, to the appearance of lactose or of galactose in the urine. If the ingestion of glucose aids in the catabolism of galactose, as was suggested by Folin and Berglund, or in the utilization of lactose, the decrease in the amounts determined by the copper methods may have been due to a decrease in the amount of one or both of these sugars in the urine. Simultaneously, there may have been an increased excretion of some other substance which has a higher glucose equivalent by the picrate methods than by the copper methods. As little as 0.2 or 0.3 gm. of a disaccharide would secure this result. This is from 0.1 to 0.15 per cent of the amount of glucose ingested. This² showed the calculated rotation, and was supposedly pure, but the presence of so small an amount of impurity could not be excluded by any examination possible in this laboratory.

However, since the excretion of sugar on the following day did not return to the level of that obtaining before the ingestion of glucose but remained the same as on the experimental day and since the excretion on both these days was nearly identical with that observed 2 days before the administration of glucose, it is probable that the slight changes observed were purely fortuitous. In any event, there was no indication of the appearance of glucose in the urine. The contrast with the effect obtained after the ingestion of glucose on a fat-protein diet was striking.

² We are indebted to the Corn Products Refining Company for the pure glucose used in this work.

In the next series of experiments (Table IV), the diet, which was the same every day, consisted chiefly of potatoes, bread, and butter, and contained approximately 300 cc. of milk and 140 gm. of fat. The excretion of sugar was approximately what it

TABLE IV.

Excretion of Sugar on a Diet Consisting Chiefly of Bread and Butter, with Effect of Certain Added Substances.

Date.	Urine.						Uric acid.	Phosphorus.	Remarks.
	Volume.	Nitrogen.	Sugar.						
			Benedict NaOH.	Folin- Berglund	Benedict Na ₂ CO ₃ .	Shaffer- Hartmann.			
1922	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Dec. 5	1,020	11.75	0.714	0.455	0.641	0.403			
" 6	1,410	10.80	0.749	0.487	0.588	0.384	0.526	0.670	
" 7	Lost.								
" 8	930	9.56	1.02	0.706	1.105	0.616	0.333	1.102	100 gm. grape nuts with each of 3 meals.
" 9	860	10.18	0.729	0.457	0.622	0.418	0.441	0.808	
" 10	910	10.00	0.620	0.443	0.706	0.444	0.642	1.054	11.9 gm. thymus nucleic acid, in 3 doses.
" 11	960	10.60	0.719	0.494	0.710	0.384	0.610	1.067	
" 12	1,440	11.15	0.712	0.464	0.630	0.390	1.286	1.315	12.3 gm. yeast nu- cleic acid, in 3 doses.
" 13	1,300	10.06	0.665	0.450	0.540	0.355	0.718	1.006	
" 14	1,320	10.25	0.555	0.479	0.512	0.408	0.630	1.030	25 gm. maltose with each of 3 meals.
" 15	960	9.77	0.550	0.418	0.564	0.416	0.540	0.837	
" 16	900	9.63	0.806	0.497	0.627	0.432	0.531	0.544	71 gm. glucose with each of 3 meals.*

Diet.

Breakfast: Cocoa, containing 200 cc. of milk, 2 rolls and butter.

Lunch: 400 cc. of weak coffee (made from dried extract, uniform every day), 50 cc. of evaporated milk, bread and butter.

Dinner: 2 eggs, boiled potatoes, bread and butter.

* At 9.30 p.m. absent-mindedly ate 2 dried figs and 2 dried dates.

had been during the first two series of experiments. On the 5th day, 100 gm. of grape nuts were substituted for about the same quantity of bread. The urine of the period was lost and the experiment was continued for another day. The excretion of sugar was increased by about 225 mg. if the figures obtained by the copper reduction methods are used, by 270 mg. with the NaOH-acetone-picrate method, and by 520 mg. by the sodium carbonate-picrate method. The return to the normal was prompt, as is shown by the figures for the next day's excretion. On the 2nd day, 11.9 gm. of thymus nucleic acid³ were dissolved in sodium bicarbonate and divided into three portions, one taken with each meal. The excretion of uric acid and of phosphoric acid was increased, but there was no change in the amount of sugar in the urine. On the 2nd day thereafter, 12.3 gm. of yeast nucleic acid³ were similarly ingested. The increase in the excretion of uric acid and of phosphoric acid was even more marked than it had been in the preceding experiment but, again, there was no increase at all in the amount of sugar in the urine.

Since grape nuts contain approximately 17 per cent of maltose¹ it was possible that the increased urinary sugar was due to the escape of some maltose. Accordingly, 25 gm. of pure maltose hydrate were taken with each of three meals. The picrate methods showed a slight decrease; the copper methods, a slight increase. Both may be disregarded. Finally, on the last day, 71 gm. of glucose were taken with each meal. All the methods showed a slight increase, which was least with the Shaffer-Hartmann method and greatest with the sodium hydroxide-acetone-picrate method. Since glucose added to urine is quantitatively recovered by all four methods, the difference may indicate the presence of some other carbohydrate following the ingestion of this amount of this preparation of glucose. However, it is possible that this sugar was derived from the two dates and two figs that were eaten.

³ The thymus and yeast nucleic acids were obtained from Dr. P. A. Levene, to whom we wish to express our sincere thanks.

Sugar Excretion and Glucose Tolerance in a Dog upon Various Diets.

These experiments were paralleled by a similar series upon a dog, weighing 17 kilos. The results are summarized in Table V. The animal was not catheterized and the periods overlap somewhat, but the error due to this is negligible. All the food was given at one time, about 9 a.m. Except as otherwise indicated, the 48 periods were consecutive. At first, the dog was fed entirely on hashed beef heart. In the next period, most of this was replaced by an isodynamic quantity of lard. According to the method used for the determinations, the amount of reducing substance in the urine decreased, increased, or remained unaffected. In the succeeding periods (Nos. 3 to 10, inclusive), the lard and then almost all the beef heart were replaced by increasing amounts of cracker meal. The amount of reducing substance in the urine was definitely, though only slightly, increased. If this increase were due to an increased excretion of glucose produced by an increased concentration of glucose in the blood during the digestion and absorption of the carbohydrate, it should have become much more evident when glucose was substituted for the cracker meal. As is evident from an inspection of the figures for Periods 11 to 17, there was no such effect, although as much as 10 gm. of glucose per kilo of body weight were fed. The amount of reducing substance in the urine, as determined by the Folin-Berglund method, was slightly increased; as determined by the Benedict and Osterberg sodium carbonate method, was slightly decreased; and as determined by the other two, remained unchanged. The same increase in the excretion of sugar following the ingestion of grape nuts as was observed in the human experiment was seen in Period 19 and, again, it was shown that this increase was not due to the maltose content of this food.

The striking loss of glucose tolerance of the human subject upon a fat-protein diet suggested a repetition of the experiment on the dog. The figures obtained in Periods 25 to 28, inclusive, show the effect of the *substitution* of 10 gm. of glucose per kilo for an isodynamic quantity of fat when the animal was upon a diet consisting chiefly of fat. There was a slight increase in the

TABLE V.

Sugar Excretion in the Urine of a Dog upon Various Diets from September, 1922 to January, 1923.

Weight of dog, 17 kilos.

Period No.	No. of days in period.	Food.	Urine.				
			Nitrogen.	Sugar.			
				Benedict NaOH.	Folin-Berglund.	Benedict Na ₂ CO ₃ .	Shaffer-Hartmann.
			gm.	gm.	gm.	gm.	gm.
1	8	885 gm. beef heart.	16.8	0.412	0.205		0.251
2	3*	240 " heart, 80 gm. lard.	6.17	0.347	0.239	0.279	0.115
3	6	240 " " 60 " " 50 gm. cracker meal.	5.05	0.259	0.158	0.210	0.143
4	2	240 gm. heart, 40 gm. lard, 100 gm. cracker meal.	5.34	0.228	0.233	0.247	0.158
5	4	240 gm. heart, 20 gm. lard, 150 gm. cracker meal.	6.52	0.391	0.246	0.302	0.157
6	3	240 gm. heart, 200 gm. cracker meal.	6.85	0.346	0.238	0.349	0.160
7	3	200 " " 210 " " "	5.33	0.280	0.217	0.283	0.153
8	4	160 " " 250 " " "	7.02	0.415	0.276	0.350	0.201
9	2	120 " " 260 " " "	7.46	0.400	0.280	0.358	0.219
10	4	80 " " 270 " " "	5.82	0.415	0.309	0.396	0.225
11	3	160 " " 250 " " "	5.98	0.370	0.288	0.362	0.203
12	3	160 " " 233 " " "	6.45	0.405	0.335	0.363	0.222
		17 gm. glucose.					
13	3	160 gm. heart, 216 gm. cracker meal, 34 gm. glucose.	6.26	0.411	0.333	0.342	0.215
14	3	160 gm. heart, 199 gm. cracker meal, 51 gm. glucose.	6.19	0.383	0.294	0.324	0.233
15	2	160 gm. heart, 165 gm. cracker meal, 85 gm. glucose.	5.56	0.343	0.276	0.341	0.207
16	3	160 gm. heart, 131 gm. cracker meal, 119 gm. glucose.	5.45	0.393	0.343	0.305	0.213
17	2	160 gm. heart, 80 gm. cracker meal, 170 gm. glucose.	4.83	0.373	0.386	0.299	0.216
18	5	80 gm. heart, 270 gm. cracker meal.	5.12	0.343	0.312	0.342	0.226
19	3	80 " " 270 " grape nuts.	6.21	1.142	0.817	1.156	0.647
20	1†	80 " " 270 " cracker meal,	6.00	0.380	0.304	0.300	0.223
21	1	80 " " 235 " " "	5.36	0.395	0.278	0.328	0.210
		35 gm. maltose.					
22	1	80 gm. heart, 235 gm. cracker meal, 35 gm. maltose.	7.79	0.508	0.469	0.486	0.332
23	1	80 gm. heart, 200 gm. cracker meal, 70 gm. maltose.	2.53	0.204	0.158	0.180	0.113
24	1	80 gm. heart, 270 gm. cracker meal.	7.91	0.566	0.519	0.628	0.390
Average of last four			5.90	0.418	0.358	0.406	0.261
25	2	160 gm. heart, 110 gm. lard.	6.14	0.398	0.356	0.325	0.207
26	1	160 " " 35 " " 170 gm. glucose.	5.17	0.621	0.506	0.453	0.430

TABLE V—*Concluded.*

Period No.	No. of days in period.	Food.	Urine.				
			Nitrogen.	Sugar.			
				Benedict NaOH.	Folin- Berglund.	Benedict Na ₂ CO ₃ .	Shaffer- Hartmann.
			gm.	gm.	gm.	gm.	gm.
27	2	160 gm. heart, 110 gm. lard.	5.56	0.429	0.344	0.308	0.221
28	1	160 " " 110 " " 170 gm. glucose.	5.75	1.07	1.03	1.03	0.881
29	2†	600 gm. heart.	12.28	0.387	0.278	0.354	0.215
30	1	600 " " 170 gm. glucose.	7.65	0.317	0.187	0.330	0.174
31	1	600 " "	11.54	0.283	0.254	0.215	0.133
32	6	Heart and casein.	12.08	0.389	0.214	0.285	0.168
33	9	Beef spleen, boiled.	19.48	0.458	0.334	0.378	0.217
34	2§	1,200 gm. beef kidney.	21.99	0.457	0.343	0.376	0.241
35	1	1,200 " " 170 gm. glucose.	24.32	0.771	0.521	0.583	0.338
36	1	1,000 gm. beef kidney.	20.00	0.477	0.359	0.369	0.235
37	2¶	Cooked beef pancreas, equivalent of 700 gm. fresh.	16.17	0.413	0.382	0.348	0.221
38	1	Cooked beef pancreas, equivalent of 700 gm. fresh, 170 gm. glucose.	13.15	0.422	0.410	0.270	0.213
39	2	80 gm. heart, 270 gm. cracker meal.	6.66	0.420	0.333	0.372	0.210
40	1	Same, plus 170 gm. glucose.	6.76	0.445	0.366	0.474	0.239
41	2	80 gm. heart, 270 gm. cracker meal.	6.99	0.464	0.417	0.415	0.246
42	3**	80 " " 220 " toasted cracker meal.	6.54	0.513	0.391	0.655	0.340
43	3	80 gm. heart, 220 gm. cracker meal.	6.94	0.435	0.304	0.360	0.211
44	2††	Washed fibrin, <i>ad libitum</i> .	11.57	0.289	0.236	0.209	0.108
45	1	170 gm. glucose, washed fibrin, <i>ad libitum</i> .	14.71	0.478	0.297	0.348	0.197
46	1††	90 gm. lard, 35 gm. pressed fibrin.	5.49	0.372	0.314	0.259	0.177
47	1	Same, plus 170 gm. glucose.	3.74	2.34	2.28	2.35	2.02
48	1	90 gm. lard, 35 gm. pressed fibrin.	1.42	0.123	0.119	0.104	0.064

* Last 3 of 9 days on this diet.

† 2nd day on this diet.

‡ Last 2 of 4 days on this diet.

§ Last 2 of 4 days, preceded by an experiment similar to that of Periods 34 to 36 but in which specimens were lost.

¶ Last 2 of 4 days, preceded by a 3 day attempt to feed fresh pancreas in adequate amounts.

|| Last 2 of 5 days, preceded by a 3 day attempt to feed beef heart and gelatin.

** Last 3 of 6 days, preceded by a repetition of the experiment included in Periods 39 to 41 and by a 2 day unsuccessful attempt to feed beef heart and starch.

†† Last 2 of 8 days on this diet, after 10 days miscellaneous experiments.

‡‡ Last day of 7 on this diet.

excretion of reducing substances. But, a few days later, when the glucose was *added* to the same fat-protein diet, there was a much greater increase in the amount of sugar in the urine.

Can this effect be produced by any kind of a carbohydrate-free diet? The next experiment (Periods 29 to 31) was planned to answer this question. 10 gm. of glucose per kilo were *added* to the basal ration of 600 gm. of beef heart. The excretion of reducing substances was decreased on the day the glucose was administered, but fell even lower on the following day. However, the tolerance was unquestionably better than it had been on the fat-rich diet. An attempt was made to secure a high protein, fat-poor diet by the use of mixtures of beef heart and casein, but the dog refused to eat these regularly.

The failure to observe any increase in the excretion of sugar in man following the ingestion of yeast or thymus nucleic acid, suggested the feeding of glandular materials, rich in nucleic acid. In spite of the fact that huge quantities were eaten, the nitrogen in the urine rising to over 20 gm. per day, or 1.2 gm. per kilo, the amount of reducing substance in the urine remained unchanged. Evidently, none of this was derived from the nucleic acid of the food.

The usual amount of glucose, added to the kidney diet, was followed by only a slight increase in the amount of sugar in the urine. According to published analyses (20, 21), beef kidneys yield from 0.8 to 1.3 calories per gm. The energy equivalent of the kidneys ingested on the day of the glucose experiment and on each of the 4 preceding days was, accordingly, at least 1,000 calories, or at least 30, and probably more than 50, per cent in excess of the animal's requirements. The glycogen stores of the animal must have been well filled at the time the glucose was given and even the fat-forming and fat-storing mechanisms must have been occupied. It is not surprising that a little glucose escaped into the urine. In a subsequent experiment (Periods 44 and 45), in which a pure protein was fed in amounts sufficient to supply not more than one-half of the energy requirements, the ingestion of the same amount of glucose was followed by a much smaller increase in the excretion of sugar on the urine.

When the glucose was added to a diet of 700 gm. of pancreas, there was no effect upon the excretion of sugar. There seem to

be only three published analyses of pancreas. Plimmer (22) found the lean of the beef pancreas to contain 17.6 per cent of protein and 7.3 per cent of fat. Powick and Hoagland (21) report 15.1 per cent of protein and 11.1 per cent of fat in calf pancreas. The third analysis is of human pancreas by Magnus-Levy (23) who found 15.6 per cent of protein and 10.6 per cent of fat. The 700 gm. of pancreas probably furnished about 115 gm. of protein and 70 gm. of fat. The failure to obtain any increase at all in the amount of urinary sugar after adding glucose to this diet may have been due to the absorption of some insulin or a precursor thereof.

In the earlier experiments, glucose had been *substituted* for the cracker meal of the carbohydrate diet. It was now (Periods 39 to 41) *added* to such a diet. The effect on the excretion of reducing substances was negligible. The experiment was repeated with the same result. Evidently, a previous diet that is free from carbohydrate, particularly if rich in fat, lowers the tolerance for glucose.

In order to test again the claim of Folin and Berglund that the sugar of the urine is derived, in part, from carbohydrates made inassimilable during cooking, the cracker meal of the diet was substituted by the same amount that had previously been heated in an oven at 140°, to a light brown color, like that of toast, and not nearly so dark as that of grape nuts. There was no control fore period, but the return to the unheated cracker meal was followed by a prompt decrease in the amount of sugar excreted. Unquestionably, the toasting had lessened the assimilability of some of the carbohydrate.

The dog was then fed on washed fibrin, with salt and bone ash added. The addition of 170 gm. of glucose resulted in a very slight increase in the amount of reducing substance in the urine.

Finally, the animal was placed on a submaintenance diet consisting almost entirely of fat. He received 90 gm. of lard and 35 gm. of moist fibrin, pressed out by hand, every day. After 7 days on this diet, when the carbohydrate stores of the body should have been largely depleted and when much of the stored body fat should have been utilized, the same 170 gm. of glucose were added to the food. The increase in the amount of sugar was the greatest yet observed, 2 gm. Evidently, the lowered

glucose tolerance is not due to any plethora of food, or to the strain upon the pancreas caused by the digestion of fat or protein, but to some other change within the organism. The nature of this change will be considered subsequently.

TABLE VI.

Effect of Glucose Taken with Fat or with Protein Meals upon the Excretion of Sugar and Nitrogen.

Subject I. G.

Total.					Per hour.				Remarks.
Urine.					Urine.				
Date.	Volume.	Nitrogen.	Sugar.		Volume.	Nitrogen.	Sugar.		
			Benedict NaOH.	Folin-Berglund.			Benedict NaOH.	Folin-Berglund.	
1924	cc.	gm.	gm.	gm.	cc.	gm.	gm.	gm.	
Oct.									
6.20-8.20 a.m.	168	1.325	0.0457	0.0336	84	0.663	0.0229	0.0168	
8.20-10.20 a.m.	500	0.605	0.0750	0.0450	250	0.303	0.0375	0.0225	8.20 a.m. Drank mixture of 233 gm. cream (40 per cent fat), 100 gm. glucose, and 500 cc. water.
10.20-12.20 p.m.	460	1.113	0.0768	0.0485	230	0.557	0.0384	0.0243	
6.35-8.35 a.m.	100	0.985	0.0620	0.0390	50	0.493	0.031	0.0195	
8.35-10.35 a.m.	160	0.972	0.0520	0.0426	80	0.486	0.0260	0.0213	8.35 a.m. Ate 367 gm. hard boiled egg white. Drank solution of 100 gm. glucose in 500 cc. water.
10.35-12.35 p.m.	104	0.995	0.0620	0.0401	52	0.498	0.0310	0.0201	

TABLE VI—*Concluded.*

Total.					Per hour.				Remarks.
Urine.					Urine.				
Date.	Volume.	Nitrogen.	Sugar.		Volume.	Nitrogen.	Sugar.		
			Benedict NaOH.	Folin-Berglund.			Benedict NaOH.	Folin-Berglund.	
	cc.	gm.	gm.	gm.	cc.	gm.	gm.	gm.	
1924									
Oct.									
7.30-8.30 a.m.	54	0.417	0.0196	0.0152	54	0.417	0.0196	0.0152	
8.30-10.30 a.m.	178	0.928	0.0524	0.0331	89	0.464	0.0262	0.0166	8.30 a.m. Ate 208 gm. hard boiled egg yolks. Drank solu- tion of 100 gm. glucose in 500 cc. water.
10.30-12.30 p.m.	102	0.989	0.0508	0.0339	51	0.495	0.0254	0.0170	
12.30-1.30 p.m.	114	0.810	0.0369	0.0225	114	0.810	0.0369	0.0225	12.30 p.m. Drank 250 cc. water.
1.30-2.30 p.m.	200	0.608	0.0354	0.0167	200	0.608	0.0354	0.0167	

Effect of Single Meals of Protein or Fat and Protein upon Glucose Tolerance.

The same conclusion is to be drawn from a series of experiments upon I. G. (Table VI). After a night's fast, the urine was collected for a 2 hour control period, during which time the subject walked to the laboratory, a distance of 1.5 miles, and went about his usual work. At the end of this time, a mixture of 233 gm. of cream, containing 93 gm. of butter fat, and 100 gm. of glucose in 500 cc. of water was taken. The nitrogen excretion during the next 2 hours was less than half of what it had been during the control period, indicating a change from a metabolism of protein and fat to one of carbohydrate. The

sugar excretion was slightly increased and remained at this level during the next 2 hours, during which the nitrogen excretion increased. If we assume that excretion of reducing substances was continued after the meal at the same rate, even if only in proportion to the amount of nitrogen excreted, the excess sugar was 92.6 mg., as determined by the method of Benedict and Osterberg and 50.8 mg., as determined by that of Folin and Wu. The relation between the two is almost exactly that obtained by the same methods upon pure lactose solutions (24). Quite probably, some of the 10 gm. of lactose taken with the cream escaped into the urine.

In a similar experiment, the control period was followed by a meal of the whites of twelve hard boiled eggs, weighing 367 gm. and containing 45 gm. of protein and 0.73 gm. of fat (20), followed immediately by 100 gm. of glucose in 500 cc. of water. The excretion of sugar and of nitrogen in the following two 2 hour periods remained absolutely unchanged.

In a third experiment, the control period was only 1 hour long and was followed by a meal of twelve hard boiled egg yolks, weighing 208 gm. and containing 33 gm. of protein and 69 gm. of fat (20). This was accompanied by 100 gm. of glucose in 500 cc. of water. The urine was collected for two 2 hour periods and for two of 1 hour each. There was a slight increase in the amount of sugar excreted but, if the amount derived from endogenous sources or from protein be calculated as proportional to the nitrogen excretion, there is an increase of only 20 mg., as determined by the method of Benedict and Osterberg, and a decrease of 15 mg., if the figures obtained by the method of Folin and Berglund are chosen. Even if the deduction is made at the hourly rate of excretion during the control period, the excess during the 6 hours following is only 15 or 39 mg., according to the method used for analysis.

It is possible that the glucose tolerance had been lowered in one or more of these experiments, but it certainly had not been affected to anything like the extent observed after several days on a fat-protein diet.

Effect of Turpentine Injections on Sugar Excretion.

The experiments upon both man and dog had shown clearly that, on a diet free from carbohydrate, the excretion of reducing

substances might be as great as on a diet containing considerable amounts of carbohydrate. The experiments had also shown that this excretion was not increased by the ingestion of nucleic acids or material rich in nucleic acids. The reducing substance was evidently not derived from exogenous nuclein metabolism. Was it derived from endogenous nuclein metabolism?

To answer this question a dog was placed upon a diet of beef heart, lard, and bone ash. The urine was collected daily and analyzed for nitrogen, uric acid, phosphorus, and reducing substances; and the ratio of the amounts of these to the nitrogen excreted was calculated. After several days observation, turpentine was injected subcutaneously in each of two places. As is evident from the figures in Table VII, there was an increase in the excretion of all these constituents (except that of phosphate on the 1st day) and an increase in the ratio of the amount of reducing substances to that of total nitrogen. After 4 days, the abscesses were opened and drained. They filled again and required emptying on each of the 2 days immediately following. During this period, the excretion of all the substances determined decreased and the ratio of reducing substances to nitrogen returned to very nearly the value found in the control period. A small amount of pus accumulated and was removed on the 2nd day of the following period. The nitrogen excretion was still high but the amount of reducing substances had decreased to the initial level. In the following period, there was a further decrease so that, at the close of the experiment, the ratio of reducing substances to nitrogen had fallen to somewhat below the value found in the fore period.

The increased excretion of reducing substances after the injection of turpentine was distinct, but it can scarcely be regarded as having been due to the increase in nuclein metabolism. The change was far too slight, unless it be assumed that most of the carbohydrate set free in nuclein metabolism was oxidized, as was most of the uric acid. If this be the case, the greater part of the reducing substances excreted during the fore period could not have come from nuclein metabolism. The amounts are too large.

It seems more probable that the slight increase in the reducing power of the urine was due to the presence of that readily decomposable conjugate glycuronic acid, that Schmiedeberg (25) found in the urines of dogs after the injection of turpentine. This

TABLE VII.

Effect of Turpentine Injection upon the Excretion of Nitrogen, Phosphorus, Uric Acid, and Sugar in the Urine of a Dog Weighing 9 Kilos upon a Diet of 135 Gm. of Beef Heart, 50 Gm. of Lard, and 15 Gm. of Bone Ash.

Date.	Nitrogen.	Phosphorus.	Uric acid.	Sugar.				Remarks.
				Benedict NaOH.	Folin- Berglund.	Benedict Na ₂ CO ₃ .	Shaffer- Hartmann.	
1923	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Nov. 1	4.00	0.390	0.021	0.177	0.136	0.121	0.075	
" 2	4.14	0.363	0.022	0.157	0.132	0.130	0.075	
" 3	4.22	0.393	0.021	0.160	0.145	0.147	0.080	
" 4	4.30	0.415	0.017	0.182	0.159	0.151	0.088	
Average	4.17	0.390	0.020	0.169	0.143	0.137	0.080	
Average Total N	×	100....	9.36	0.48	4.05	3.43	3.29	1.92
Nov. 5	4.95	0.281	0.025	0.279	0.220	0.276	0.151	1.5 cc. turpentine in each of two places, subcutaneously.
" 6	6.42	0.588	0.029	0.335	0.253	0.356	0.202	
" 7	5.96	0.410	0.046	0.300	0.239	0.253	0.166	
" 8	5.70	0.358	0.033	0.261	0.192	0.273	0.156	
Average	5.76	0.409	0.033	0.294	0.226	0.290	0.161	
Average Total N	×	100....	7.10	0.57	5.26	3.92	5.04	2.80
Nov. 9	5.59	0.345	0.036	0.260	0.176	0.233	0.142	Both abscesses op- ened, 85 cc. pus obtained.
" 10	6.40	0.371	0.060	0.271	0.193	0.254	0.140	
" 11	4.42	0.328	0.041	0.204	0.140	0.144	0.082	
Average	5.47	0.345	0.045	0.245	0.170	0.210	0.121	
Average Total N	×	100....	6.31	0.82	4.48	3.11	3.84	2.21
Nov. 12	5.50	0.343	0.039	0.201	0.149	0.174	0.093	Both abscesses op- ened, 15 cc. pus obtained.
" 13	4.78	0.384	0.026	0.166	0.144	0.135	0.085	
Average	5.14	0.364	0.033	0.184	0.147	0.155	0.089	
Average Total N	×	100....	7.08	0.64	3.58	2.86	3.01	1.73
Nov. 14	4.46	0.404	0.023	0.171	0.122	0.113	0.078	Abscesses healed.
" 15	4.21	0.389	0.018	0.168	0.125	0.113	0.071	
Average	4.34	0.397	0.020	0.170	0.124	0.113	0.075	
Average Total N	×	100....	9.15	0.46	3.92	2.86	2.60	1.73

substance, in solution, decomposed on standing at room temperature for a short time and these solutions then reduced alkaline copper solutions. This view is confirmed by the fact that the increased excretion of reducing substance practically ceased after the abscesses had been emptied the first time, thus removing the unabsorbed turpentine. The abscesses filled rapidly and the excretion of nitrogen and uric acid continued high, but that of reducing substances was only very little above the level observed in the fore period. Certainly, these experiments afford no support for the view that the reducing substances of the urine are derived from endogenous nuclein metabolism.

Other Possible Sources of the Urinary Sugar.

The cerebroside contains galactose, but the amounts of galactose that could conceivably be liberated in their catabolism should be well within the capacity of the organism to convert them into glycogen or into glucose. We seem to be driven to accept the proteins as the source of the urinary sugar.

Since small doses of glucosamine, and part of larger amounts, administered to men and animals, always disappear (26), and are apparently metabolized, it is unlikely that this substance is, under ordinary conditions, present in the urine. Apparently, chondrosamine has never been administered to men or animals. It is possible, but scarcely probable, that it may be present in the urine. It is difficult to imagine that either glucosamine or chondrosamine, or both, could account for the amount of reducing substances in the urine on a fat-protein diet.

One of us (27) has recently reviewed the literature on pentosuria, from which it appears that the pentose is derived from protein. It is possible that some of this pentose always escapes into the urine.

The concentrated preparations of the reducing substances from normal urine yield large quantities of furfural when heated with hydrochloric acid, but we are not yet certain that we have entirely eliminated glycuronic acids as the source of this furfural. We hope to continue the work.

DISCUSSION.

During the course of this work, other observers also reported a lowered tolerance to glucose in men and dogs upon a carbo-

hydrate-poor diet. Kageura (28) found that the hyperglycemia in men following the ingestion of the Sakeguchi test meal (100 gm. of rice, 2 eggs) was greater after a carbohydrate-poor diet than after a diet rich in carbohydrates. In some cases, the addition of 100 gm. of rice to a meat-fat diet was followed by glycosuria.

Similar results were obtained with dogs, using from 5 to 8 gm. of glucose per kilo as the test meal. Other experiments upon dogs (29) indicated that protein and fat were equally effective in lowering the tolerance to glucose. In that respect, his results differed from ours, but examination of the reports of his experiments shows that what Kageura called a protein diet consisted largely of fat. The amount of nitrogen excreted in the urine is not given, but the statements as to the character of the diet indicate that about 5 per cent of the calories (9 per cent in one experiment) were derived from carbohydrate and that not more than 35 per cent were derived from protein. Since the energy equivalents of the diets seem to have been rather less than the requirements of the dogs, it is certain that fat furnished at least 60, and probably more than 65, per cent of the calories *metabolized*. The diet in the control periods (so called fat diet) consisted of 100 gm. of fat beef for dogs weighing 10, 11, and 14 kilos. This could have furnished not more than two-thirds of the energy requirements and, probably, even less of the nitrogen requirement. It appears quite certain that, in these periods, not more than about 80 per cent of the metabolism was at the expense of fat.

The experiments reported on this question in the present paper were performed upon only one dog, but they seem to show quite definitely that a diet consisting only of protein does not lower the tolerance for glucose to anything like the extent that does a diet chiefly of fat. Moreover, the fact that the glucose excretion was so much greater in Period 28 than it was in Period 30, in both of which 170 gm. of glucose were added to the previous diet, indicates that the diet in the latter experiment was less injurious to the glucose tolerance than was that in the former. Since the beef heart used contained at least 15 per cent of fat, and probably as much as 20 per cent, the fat content of the two diets was not so greatly different, about 140 gm. in the former and 100 or 120 gm. in the latter. The additional amount of

protein in the latter experiment appears to have had a beneficial, rather than a detrimental, effect upon the glucose tolerance. And even with the very great nitrogenous metabolism in the experiment of Periods 34 to 36, the glucose tolerance remained high. It was least in the last experiment, in which the metabolism was almost entirely at the expense of fat.

An increased hyperglycemia, after the ingestion of 50 gm. of glucose, was observed by Southwood (30) in men who had been on a carbohydrate-free and, apparently, submaintenance diet for 36 hours before the test. In some cases there was evident glycosuria. Similar results were obtained after a mixed meal following a period of carbohydrate-free food. In the one experiment performed, the subcutaneous administration of 3 units of insulin at the same time as the glucose was followed by a normal hyperglycemic curve (like that obtained on the same individual after taking glucose when on a mixed diet). There was no glycosuria, although this individual had regularly shown this after the ingestion of glucose when on a carbohydrate-free diet, without insulin.

Although Folin and Berglund⁴ regard the hypoglycemia that follows the hyperglycemia after glucose ingestion as "an index of the fact that the need for sugar transportation from some tissues to others has temporarily fallen very low or ceased altogether," it is more generally (McLean and de Wesselow (31) and Foster (32)) considered to be due to an "overactivity" of the glycogenic mechanism, which has been stimulated by the hyperglycemia. Foster found that there was no increase in the concentration of glucose in the venous blood following the ingestion of a second dose of 100 gm. of glucose when the concentration of sugar in the blood had just returned to the normal after the first dose. These results can scarcely be explained except as an indication of overstimulation of the glycogenic mechanism by the first dose.

Robertson (33) regards Southwood's results, which have already been discussed, as further evidence that alimentary hyperglycemia stimulates the pancreas and that absence of such stimulation for 36 hours reduces the functional capacity of the islet tissue of the pancreas.

Some of Kageura's results indicate that other organs are certainly involved in this loss of the glycogenic capacity of the

⁴ Folin and Berglund (3), p. 267.

organism. He found (34) that the livers of the dogs on the carbohydrate-poor diet and of those on the carbohydrate-rich diet, if removed after a 20 hour fast, contained about the same concentration of glycogen. Nevertheless, the former, when perfused with a mixture of blood, glucose, and salt solution, formed much less glycogen than did the latter. This difference was not due to any difference in the bloods, for the same difference in glycogenic function was observed when the perfusing bloods were exchanged; that is, when the liver of a dog that had been on the carbohydrate-poor diet was perfused with a mixture containing blood from a dog that had been on a carbohydrate diet, and *vice versa*.

These results might be explained by assuming that the concentration of glucose in the blood determines the rate of the liberation of insulin by the pancreas. When much insulin is liberated, a considerable amount is stored in the liver. When little is liberated, the concentration in the liver falls. A dog fed a fat-protein diet does not have his pancreas stimulated by hyperglycemia, his liver contains little insulin and, therefore, cannot form glycogen as rapidly as does the liver of a dog that has been kept on a carbohydrate diet.

While we see no reason why hyperglycemia should not be regarded as a stimulus to increased production, or liberation, of insulin by the pancreas, we must point out that this hypothesis fails to explain why, in our experiments, a fat diet should have been so much more effective in lowering the glucose tolerance than was a protein diet. Indeed, a protein diet seems to have had practically no effect, whereas a diet chiefly of fat had a very marked effect. It seems that another factor must be involved.

Action of Insulin.

As is well known, acetoacetic acid is not readily, if at all, oxidized unless glucose is also being oxidized. For the latter process and, consequently, for the former, the presence of insulin is necessary. We suggest that the function of insulin is to combine glucose, with or without previous change, with some other substance, apparently phosphate. The formation of this compound, which we will call "glucose: X," we believe to be essential to the oxidation of glucose, and to its conversion into glycogen or into fat.

It is this glucose:X that we believe to be combined with acetoacetic acid to secure the catabolism of the latter.

If an individual has been on a carbohydrate-poor diet sufficiently long to produce a ketosis, as is known to have occurred in Southwood's experiments, in those of Kageura, and in that upon I. G., which is summarized in Table II of this paper, the existence of this ketosis is, in itself, an indication that the supply of glucose:X is not sufficient for normal metabolism. Since, under these conditions, a large part of the metabolism must be taking place in the muscles, certainly not all in the liver, it is not difficult to imagine that such glucose:X as is available will be transferred to the muscles or other active tissues. If a high concentration of glucose is now thrown into the liver, there is not enough "X" there to permit of the conversion of the glucose into glycogen with the normal rapidity. More glucose than normally escapes into the systemic circulation and the concentration in the blood rises to unusually high values and there may be a very considerable excretion of glucose before the glycogenic and fat-forming organs are able to dispose of the glucose.

It is obvious that this mobilization of glucose:X will occur before the appearance of acetoacetic acid in the urine or even its increased concentration in the blood. These are only the signs that adequate amounts of glucose:X can no longer be made available.

It may be objected that some of Kageura's experiments (35) contradict this hypothesis. He found, when he repeated his carbohydrate tolerance test upon men or dogs upon a carbohydrate-poor diet, that the rise in the concentration of sugar in the blood was as high in the second experiment as it was in the first, although the concentration of the ketone bodies in the blood, before administering the carbohydrate, was lower in the second experiment than in the first. It is unfortunate that Kageura did not determine the amount of ketone bodies in the urine before administering his carbohydrate test meal. It is possible that the organism had become adapted so as to excrete ketone bodies at a lower concentration in the blood in the second experiment than in the first. Also, it is possible that fat may be oxidized without the cooperation of glucose:X. This is particularly probable in dogs, in which it is very difficult, if not impossible, to establish a ketosis comparable to that observed in man.

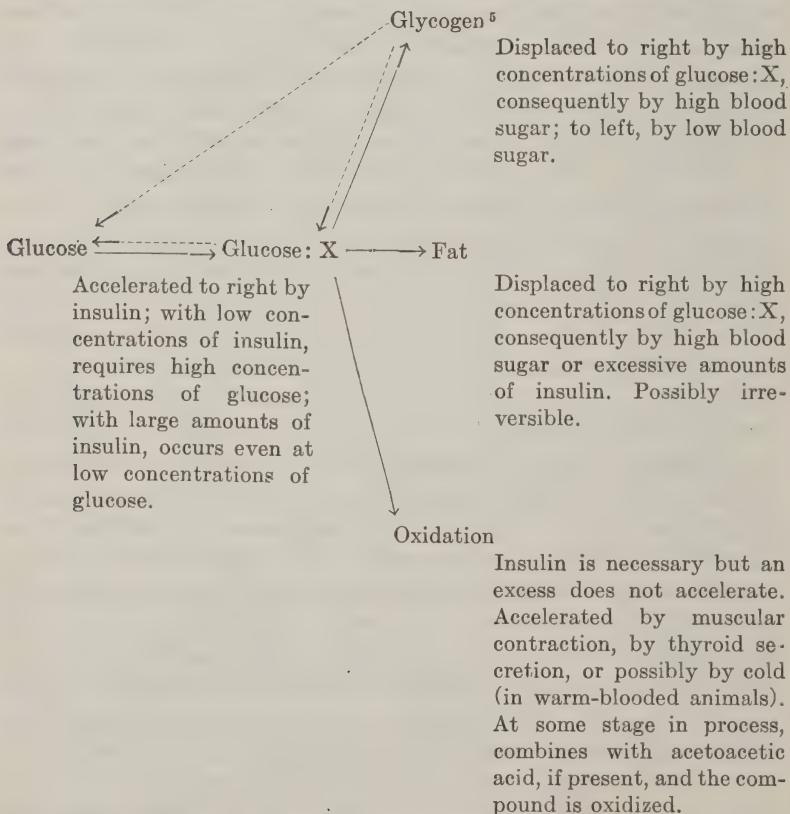
It is not within the purpose of this paper to discuss, in any great detail, the nature of the action of insulin. The subject has been reviewed by Macleod (36). But, in a few words, we wish to present the following hypothesis, suggested by our experiments. The first effect of insulin is to accelerate the formation of glucose:X; with, apparently, a decrease in the inorganic phosphate of the blood (37, 38). The subsequent fate of this compound depends upon the conditions existing within the body, particularly the concentration of glucose in the blood, the concentration of insulin, and the energy requirements of the body. If there is a demand for energy, as there is in severe diabetes, the glucose:X is oxidized, for which process insulin again appears to be required. If the demand for energy is satisfied and the concentration of insulin is not excessive, glycogen is deposited. If the concentration of glucose in the blood is low, glucose:X or glycogen will, normally, be converted into glucose in order to keep the concentration of the latter within normal limits. But, in the presence of an excess of insulin, the equilibrium conditions are disturbed. The reaction is forced in another direction. Fat formation occurs or begins. It may not be complete; in the absence of other necessary factors, it may be arrested at some intermediate stage; it may not take place in the liver exclusively or at all. Therefore, the failure of Dudley and Marrian (39) to find an increased concentration of fat in the livers of mice treated with insulin does not disprove this hypothesis. It is interesting to observe that McCormick and Macleod (40) found no appreciable difference in the glycogen deposition in the livers and skeletal muscles of normal and insulin-treated animals unless the amount of insulin was sufficient to induce convulsions or hyperexcitability. Presumably, hypoglycemia existed in these animals, yet the glycogen content of the livers was lower than in the controls. The glycogen content of the heart muscle of all the insulin-treated animals appeared to be higher than that of the controls. That may indicate that fat formation does not take place in the heart, so that overdosage with insulin can push the reaction only in the direction of glycogen, not towards fat.

The fact that patients with mild diabetes are inclined to become stout may seem to argue against the assumption that insulin accelerates the formation of fat. But we believe that this reaction proceeds even with a subnormal concentration of insulin,

possibly even in its absence, but at a higher concentration of glucose. This is the condition obtaining in mild diabetes. Through the lack of adequate amounts of insulin, the oxidation of glucose:X is checked and the concentration rises; some is converted back into glucose, directly or through the stage of glycogen, giving rise to the high blood concentration of blood sugar observed in such cases. An equilibrium is reached, after which any increase in glucose concentration drives the reaction in the direction \rightarrow glucose:X \rightarrow fat.

It is not intended, in this hypothesis, to rule out the effect of nervous impulses or of the hormones of glands other than the pancreas.

Schematically, the relationships may be expressed as follows:



⁵ The broken lines indicate alternative possibilities; either or both may occur.

SUMMARY AND CONCLUSIONS.

The sugars excreted in normal urine are made up of difficultly- or non-assimilable carbohydrates and of reducing substances derived from the protein of the food and from endogenous sources. The nature of those in the former group depends upon the diet. This group may include lactose from milk, pentoses from fruits, and caramelized sugars and dextrins. The amounts of all of these are usually small. Upon ordinary diets, at least one-half of the sugars of the urine originate from the protein of the food or from endogenous sources. Apparently, they do not arise from nuclein metabolism. It is suggested that they may be pentoses, which, it is suggested, are always formed from protein and, in pentosuria, are either formed in larger quantities or escape further catabolism.

The tolerance for glucose is distinctly lowered by a carbohydrate-free diet. The effect appears to be the greater, the larger the part that fat contributes to the metabolism. Protein appears to counteract the effect of fat. A possible explanation for the interference of fat metabolism with glycogen formation and for the part that insulin plays in these processes is developed.

Glucose may appear in the urine of normal persons after the ingestion of quite large quantities of glucose or after the ingestion of a carbohydrate meal immediately following a period of carbohydrate-free diet. Just as with any other function, there are probably all gradations in the ability of individuals to assimilate glucose. There are those who are unquestionably normal and those who are frankly diabetic, just as there are those who have unquestionably normal hearing and those who are totally deaf. Just what shall be called "normal" is a matter of statistics and of definition. Not nearly enough of the variations in the ability to assimilate glucose is as yet known.

But the existence of a "glucose threshold" seems established by the work of others (3, 8, 9). Its value varies with individuals and, probably, in the same individual at different times, and there may even be a slight variation among the millions of glomeruli and tubules that make up any one pair of kidneys, but there is no evidence whatever of the leakage of glucose through the kidney when the concentration in the blood is below a certain level. In unquestionably normal individuals, upon a mixed

or a carbohydrate diet, not containing any large quantities of glucose, the concentration of glucose in the blood probably never reaches this threshold and their urines probably never contain glucose.

Addendum.—Since the above was written, a paper by Höst (41), in which he, also, finds that the sugar in normal urine is not glucose, has appeared. He used several human subjects, so that the possible objection to the work reported by the present writers, of too few and unusual subjects, cannot be raised. The evidence upon which Höst bases his conclusions is of two kinds: (1) the properties of the urine sugar as contrasted with those of glucose; and (2) metabolism experiments, accompanied by determinations of the concentration of glucose in the blood. Höst finds that the fermentable part of the urine sugar is fermented much more slowly than is glucose added to urine. Also, although fermented urine does not yield an osazone when treated with phenylhydrazine, as does normal unfermented urine, the osazone obtained from such unfermented urine does not resemble phenylglucosazone. Typical phenylglucosazone crystals were obtained when glucose was added to fermented urine and the mixture then treated with phenylhydrazine, although the minimum amount of glucose that could be so identified was somewhat greater than the amount of sugar previously fermented. But either typical phenylglucosazone crystals, or none at all, were obtained. They never resembled those obtained from normal urine.

In the metabolism experiments, blood and urine were obtained and analyzed at frequent intervals. It was definitely shown that there was no increase in the excretion of sugar until the concentration in the blood had risen to a certain level. This level seemed to be somewhat lower in normal individuals whose tolerance was tested at 4 p.m. than in those examined at 8 or 9 a.m. A diabetic excreted more glucose after the ingestion of 6 gm. of glucose with 300 gm. of meat than after the ingestion of glucose alone. But this was not due to any antagonistic action of the external and internal secretion of the pancreas, as was suggested by Benedict, Osterberg, and Neuwirth (2), but was due to a lowered threshold for glucose after the ingestion of meat, for neither in the diabetic nor in normal individuals was there any greater hyperglycemia after glucose and meat than after glucose alone. Although the concentration of sugar in the blood was not increased to any greater extent by the ingestion of 100 gm. of bran bread than by the ingestion of the same quantity of wheat or rye breads, the amounts excreted in the urine were much larger in the former series than in the latter. Moreover, the maximum blood sugar concentration occurred during the 1st hour; whereas the maximum increase in sugar excretion usually occurred in the 3rd hour.

As a result of these experiments, Höst concludes:⁶

⁶ Höst (41), p. 412.

"We may, therefore, distinguish between two kinds of sugar excretion in the urine: 1. Physiological sugar excretion, which has no relation to the amount of glucose in the blood and which comprises sugars whose nature is not known, but which probably do not include glucose. After meals consisting of bread, as well as in urine which is concentrated, these physiological sugars may occur in such quantities that the reduction reactions commonly employed are positive. 2. Pathological sugar excretion, caused by the passage of the glucose of the blood into the urine when the blood sugar concentration exceeds the renal threshold."

We have, also, most regrettably, overlooked the important work of Staub (42). He found that the hyperglycemic reaction after the ingestion of 20 gm. of glucose was less after a fast of 10 or 15 hours than after one of 5 hours, but that it became more marked after a fast of 24 to 48 hours. The same increased hyperglycemia was obtained after 2 days on a carbohydrate-free diet and *after* severe work. *During* severe work, the hyperglycemia was less than when at rest. Staub refers to observations of Bang (43), Bøe (44), Barrenschén (45), and Bergmark (46), all of which indicate that glucose tolerance is lowered by fasting. Staub suggests that hyperglycemia is a stimulus to the liberation of assimilative enzymes, which are transferred to liver or muscle, as the need arises. This hypothesis is not essentially different, though less detailed, than the one advanced in this paper. It does not, however, consider the relation between fat and carbohydrate metabolism.

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THE ACIDOSIS OF OPERATIVE ANESTHESIA.*

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The literature of the last few years has clarified to a large extent the views held concerning the effect of general anesthesia on the acid-base balance of the animal body. Since Menten and Crile's (1) observations on the fall of pH in the blood of ether-anesthetized rabbits, considerable data have been accumulated demonstrating that an acidosis is produced during ether, chloroform, and nitrous oxide anesthesia.

The observations of Y. Henderson and Haggard (2) in 1918 showed that ether anesthesia reduced the CO₂ capacity of the blood of the dog, but they believed that this was the result of a blowing off of CO₂ due to a hyperventilation. As Van Slyke (3) has already pointed out, such a decrease in CO₂ capacity, due to CO₂ deficit alone, would have to be associated with an increased blood pH or alkalosis, while on the other hand a decrease of the CO₂ capacity due to the combination of the alkali with non-volatile acids would result in an acidosis.

The necessity of determining the pH of the blood as well as the total CO₂ content was pointed out by Van Slyke, Austin, and Cullen (4) in 1922. They reported the effect of ether anesthesia on the acid-base balance of the blood of dogs, and also, in the same paper, reviewed the literature on the subject. In their experiments on dogs the ether was administered by the drop method with a few layers of gauze. In two cases they used a trachea cannula connected to a Y-tube, valves, and ether inhaler so that rebreathing would not occur. In their six experiments the pH values were

* This paper is study No. 36 of a series of studies on metabolism from the Harvard Medical School and allied hospitals, a part of the expense of which has been paid by the Proctor Fund for the study of chronic diseases.

calculated or determined as follows: three from the $\frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3}$ ratio, and three colorimetrically. In one experiment the electrometric method was also used. In an anesthesia of approximately 1 hour duration the average pH change was found to be from about 7.44 to 7.20; the CO_2 capacity, or in some cases content, dropped from 13 to 34 volumes per cent; the CO_2 tension of the arterial blood usually increased about 20 mm. of Hg in pressure. In only one case was the CO_2 tension lowered.

Since then similar results have been reported by Leake, Leake, and Koehler (5) from a series of experiments upon etherized dogs, using the electrometric method for determining pH. Recently Cullen, Austin, Kornblum, and Robinson (6) have studied nitrous oxide and chloroform as well as ether anesthesia more extensively and have observed that there is a sudden drop in CO_2 capacity as well as in pH during the first few minutes of anesthesia; thereafter the drop is a slower one.

However, when the literature is reviewed regarding the changes in acid-base balance during anesthesia as applied to the patient in the course of an operation, we find that our knowledge is not very complete. Reimann and Bloom (7) reported a series of cases after operation and found a definite decrease of the CO_2 capacity of the blood of from 10 to 20 volumes per cent. Ether was used in every case but one in which nitrous oxide was the anesthetic. The method for administering the ether was the drop method and gauze. These workers believed that low alkali values were associated with poor postoperative conditions, such as rapid pulse, restlessness, and gas distress.

Caldwell and Cleveland (8) reported a similar drop in CO_2 capacity in a series of cases after operation.

The purpose of this study was to determine what changes occurred in the acid-base equilibrium during anesthesia and operation as carried out in the Surgical Amphitheatre of the Massachusetts General Hospital.

Methods.

The subjects for observation were patients that were handled in the usual way, the anesthesia being given by nurses trained as anesthetists. Different methods of administration were studied. The closed cone method, used nearly exclusively for ether at the present time at the Massa-

chusetts General Hospital, needs special description. A cross-section of such a cone is shown in Fig. 1. The core of the frustum of a cone is made of a fairly heavy piece of cardboard so that the height is about 6 inches with a bottom opening of approximately 3 by 5 inches. The core is covered with a double layer of towelling, the top end being packed fairly heavily with gauze. The top is then flattened, compressed, and pinned as illustrated. The cardboard at the portion of the cone fitting over the nose is cut away so that the cone fits tightly over the face. The ether is poured in from the open end of the cone so as to keep the gauze fairly well saturated. During anesthesia it is the custom to keep the cone down fairly tightly so that the subject breathes through the cone and thus in part rebreathes. In the drop method used for a few control observations the usual method was employed with twelve layers of gauze over a wire frame which fitted loosely over the face.

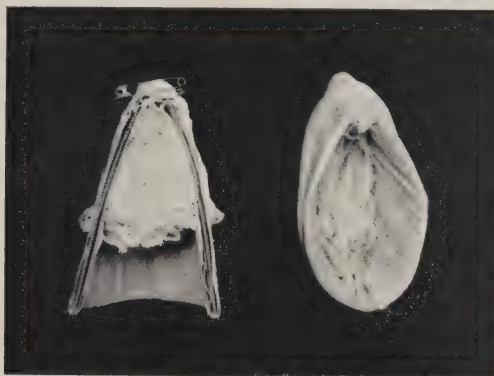


FIG. 1.

Ether was also given by means of the Connell machine where an ether-air mixture is blown into the nasopharynx by means of a nasal tube. This method gives no opportunity for rebreathing.

For nitrous oxide-oxygen anesthesia an Ohio machine was used, the amount of rebreathing being regulated by the tension of the exhaling valve.

The blood was drawn from the median basilic vein of the arm, collected directly under paraffin oil over the minimum amount of powdered potassium oxalate necessary to prevent clotting; no tourniquet was used. The blood was then immediately chilled in ice water and usually used for the determinations within 30 minutes.

The pH was determined directly on the whole blood by the electro-metric method as previously described by the author (9). The hydrogen used contained 5.5 per cent of CO_2 .

TABLE I.
Ether Anesthesia.
Closed Cone Method.

Case No.	Operation.	Duration of anesthesia.	Time blood was drawn.	pH	Total CO ₂ , <i>vol. per cent</i>	CO ₂ tension, <i>mm. Hg</i>	Remarks.
1	Drainage, appendix abscess.	1 hr. 40 min. Recovery, 1 hr. 50 min.	16 hrs. before. 4 min. after. 1 hr. after.	7.49 7.37 7.41	49.8 48.5 46.0	36.5 46.3 40.1	Age, 9. Temp. 101°F. Resp. 24-42. Pulse 120-140. Fever, rapid respiration and pulse before operation. Postoperative condition good. No nausea, vomiting, or gas distress.
2	Repair of bilateral inguinal hernia.	2 hrs. 10 min. Recovery, 1 hr. 50 min.	2 min. after. 1 hr. after.	7.22 7.41	45.0 46.0	59.8 39.8	Age, 35. Temp. 98.9°F. Resp. 22-34. Pulse 80-110. Preoperative condition, very good. Slight nausea, vomiting, and gas distress for 24 hrs. after operation.
3	Repair of left inguinal hernia.	1 hr. 10 min. Recovery, 2 hrs.	1 hr. 9 min. during. 10 min. after. 2 hrs. after.	7.09 7.12 7.30	40.0 38.2 43.1	63.4 56.9 48.4	Age, 36. Temp. 98.6°F. Resp. 26-22. Pulse 80-100. Preoperative condition, good. Postoperative condition, poor. Nausea and vomiting for 48 hrs. Became very cyanotic when ether was started so was given oxygen by tube into cone. Color good after oxygen was given. Oxygen saturation of venous blood at end of anesthesia, 86 per cent.

4	Excision of varicose veins, both legs.	2 hrs. 40 min. Recovery, 3 hrs. 20 min.	1 hr. before. 10 min. during. 1 hr. 20 min. during. 2 hrs. 30 " " 30 min. after. 1 hr. 5 min. after.	7.42 7.31 7.21 7.19 7.31 7.35	54.9 50.1 53.6 46.0 44.9 46.7	46.7 55.6 73.1 65.8 49.0 46.9	Age, 25. Resp. 18-28. Pulse 90-130. Preoperative condition, good. Postoperative condition, fair. Slight nausea and vomiting for 36 hrs. No cyanosis during anesthesia.
5	Repair of bilateral inguinal hernia.	1 hr. 50 min. Recovery, 2 hrs. 30 min.	10 min. during. 1 hr. 45 min. during. 30 min. after.* 3 hrs. after.*	7.32 7.23 7.21 7.35	45.1 44.1 41.1 46.8	48.4 57.1 56.1 47.0	Age, 52. Resp. 26-32. Pulse 80-118. Preoperative condition, good. Postoperative condition, poor. Nausea and vomiting for 20 hrs.
6	Removal of bone fragments, skull fracture.	1 hr. 30 min. Recovery, unconscious for 6 hrs.	15 min. before. 5 " after.	7.36 7.29	50.2 45.0	48.7 51.5	Age, 20. Resp. 26-32. Pulse 78-128. Preoperative condition, fair. Skull fracture 6 hrs. before, considerable hemorrhage, partly conscious at times before operation. Postoperative condition, poor. Nausea and vomiting. Connell ether pump used.
7	Repair of bilateral inguinal hernia.	2 hrs. Recovery, 1 hr.	1 hr. before. 5 min. during. 1 hr. 55 min. during. After 12 min. CO ₂ . 30 min. after CO ₂ . 60 " " "	7.43 7.35 7.21 7.14 7.34 7.39	51.2 48.4 47.6 52.9 48.0 49.0	42.7 48.6 64.9 83.8 49.1 45.0	Age, 45. Resp. 18-32. Pulse 80-140. Preoperative condition, good. Postoperative condition, good. No nausea, vomiting, or gas distress. CO ₂ inhalation given for 12 min. following anesthesia.

* Moist râles throughout both lungs, vomitus obstructing respiration.

The total CO_2 was determined in one of two ways. It appeared at first as if sufficient ether vapors, or nitrous oxide gas, were given off from the blood after anesthesia to vitiate the gasometric results. Van Slyke, Austin, and Cullen (4) reported satisfactory duplicates of the total CO_2 in etherized blood, using the gasometric and titration methods. To check this point further we used the constant volume apparatus of Van Slyke for total CO_2 , and also a titration method in all details the same as that described by Van Slyke (10) except that the titration was done electrometrically. In this way a complete titration curve could be obtained of either plasma or reduced whole blood and the pH of the original blood sample placed upon the curve. In using whole blood 10 cc. of 0.01 N HCl were used for 1 cc. of blood instead of 5 cc. as for plasma. A few duplicate determinations showed quite conclusively that there was very little difference between the two methods. Accordingly, the gasometric method was used in nearly all the total CO_2 determinations reported.

The respiratory minute volume was determined by means of a recording spirometer, the expired CO_2 being absorbed by soda-lime.

Calculation.—The CO_2 tension of the blood was calculated from the pH and total CO_2 based upon Hasselbalch's equation:

$$\text{pH} = \text{pK} + \log \frac{\text{total CO}_2 - \text{free CO}_2}{\text{free CO}_2}$$

assuming a pK value of 6.15 for whole blood. Volumes per cent of free CO_2 were converted to CO_2 tension by using the solubility coefficient of 0.0587 for whole blood at 38°C . This value was derived by assuming that the solubility of CO_2 in blood is determined by its water concentration as discussed by Van Slyke, Wu, and McLean (11). Thus with a water concentration of 81 per cent by weight of whole blood and a blood-water solubility coefficient of 0.0725 at 38°C ., the value of 0.0587 is obtained for whole blood. The CO_2 tension derived from calculations based upon whole blood is not altogether accurate for the pH measured is that of the plasma, while the fixed CO_2 includes that of the corpuscle, the reaction of which is more acid than that of the plasma. However, for comparative purposes the CO_2 tension so derived is undoubtedly sufficiently accurate, at least within the variation that venous blood is normally subjected to.

RESULTS.

Table I gives the changes observed in the acid-base balance during ether anesthesia induced by the closed cone method and

TABLE II.
Ether Anesthesia.
Open Drop Method.

Case No.	Operation.	Duration of anesthesia.	Time blood was drawn.	pH	Total CO ₂ . <i>vol. per cent</i>	CO ₂ tension. <i>mm. Hg</i>	Remarks.
1	Excision of varicose veins.	1 hr. 50 min. Recovery, 1 hr. 35 min.	1 hr. 10 min. before. 8 min. during. 1 hr. 10 min. during. 1 " 50 " " 50 min. after.	7.41 7.34 7.30 7.29 7.36	50.2 47.6 44.2 43.8 47.0	43.6 48.7 49.7 50.0 47.5	Age, 41. Resp. 20-26. Pulse 80-118. Preoperative condition, very good. Postoperative condition, good. No nausea, slight vomiting. Little distress with rapid recovery.
2	Appendectomy for chronic appendicitis.	1 hr. 30 min. Recovery, 1 hr. 25 min.	12 hrs. before. 30 min. during. 1 hr. 30 min. during.	7.44 7.36 7.31	52.1 47.3 42.1	42.6 46.0 45.9	Age, 36. Temp. 98.8°F. Resp. 23-32. Pulse 80-110. Preoperative condition, good. Postoperative condition, good. No nausea, vomiting, or gas distress.

operation. In all cases there was an acidosis ranging from blood pH 7.37 to 7.09. The available alkali of the blood, as measured by the total CO_2 , decreased, but usually only slightly and not at all

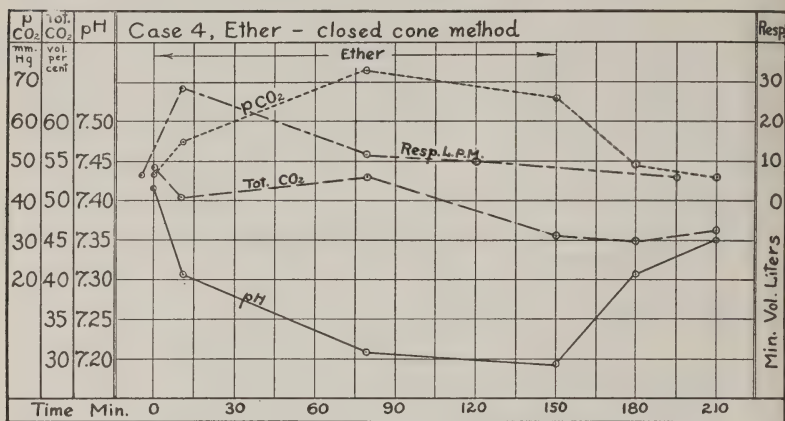


FIG. 2.

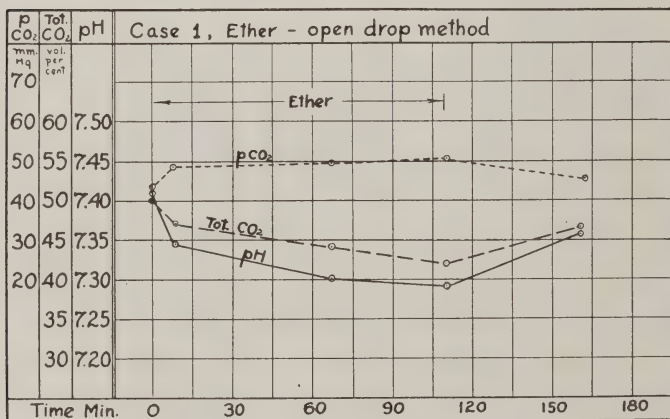


FIG. 3.

comparable to the drop in pH had the latter been caused by non-volatile acids. Table II represents the changes using the open drop method. Although there was an increase in CO_2 tension of the venous blood in all cases, it will be noticed that the increased

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Experiment No.	Subject.	Duration.	Time blood was drawn.	pH	Total CO ₂ . vol. per cent	CO ₂ tension. mm. Hg	Minute volume of respiration. l.	Respiratory rate.	Remarks.
1	A. E. K.	1 hr. 15 min.	5 min. before. 48 " during. 1 hr. 13 min. during.	7.43 7.35 7.33	53.2 55.3 55.1	44.4 55.9 57.3	6.33 30.30	17 22 23	Closed cone method. Subject conscious of some respiratory difficulty during first minutes only. Stiffing sensation soon disappeared and subject felt drowsy and dozed most of the time. Not conscious of hyperpnea unless attention was focused upon it.
2	G. M. R.	1 hr. 25 min.	7 min. before. 1 hr. 23 min. during.	7.45 7.35	54.7 57.5	43.8 57.8	7.10 28.90	15 21	Closed cone method. Response of subject very similar to above. No cyanosis or change of color in either case.
3	A. E. K.	1 hr. 30 min.	10 min. before. 58 " during. 1 hr. 27 min. during.	7.46 7.36 7.35	52.0 54.9 55.2	40.7 53.3 55.4	6.45 33.00	16 21 21	Nitrous oxide machine using 80 per cent N ₂ and 20 per cent O ₂ . Subject noticed stiffling effect at first, conscious of increased respiratory effort throughout.

tension was very much greater in the closed cone than in the open drop method. This is probably the reason for the milder acidosis in the cases done with the latter method.

The acid-base changes of ether anesthesia as produced by the closed cone and open drop methods can best be seen in Figs. 2 and 3. That the closed cone is partly responsible for the lower pH and higher CO_2 tension is further shown in Table III, Experiments 1 and 2, and also graphically in Fig. 4. This shows the changes in the blood and the great increase in minute volume respiration produced by the cone alone without ether on a normal

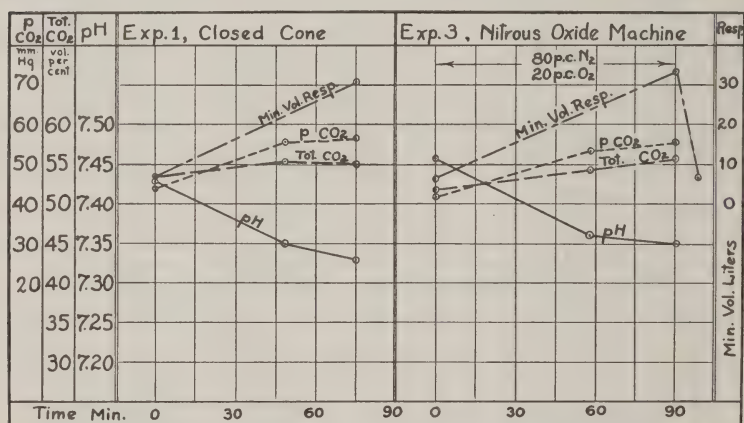


FIG. 4.

individual. The cone was held firmly in place for 1 hour and 15 minutes in the experiment charted.

Case 7, Table I, illustrates the effect of CO_2 inhalation after anesthesia using the Y. Henderson inhaler. The changes are best shown graphically in Fig. 5.

The blood changes produced by nitrous oxide anesthesia are shown in Table IV and a typical case is illustrated graphically in Fig. 6. The effect of the nitrous oxide-oxygen machine alone in causing rebreathing on a normal individual was determined by substituting nitrogen instead of nitrous oxide. The results are given in Table III, Experiment 3, and also in Fig. 4.

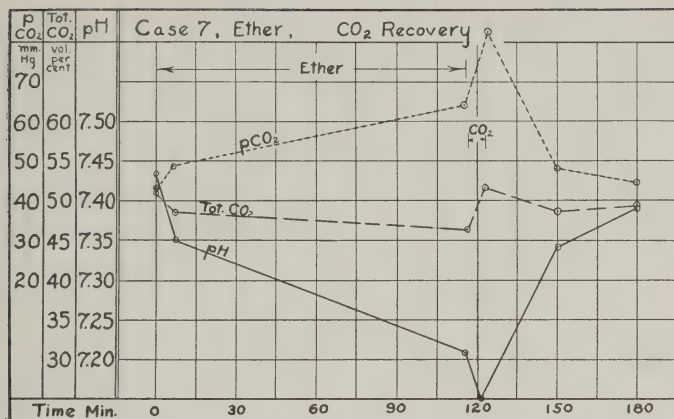


FIG. 5.

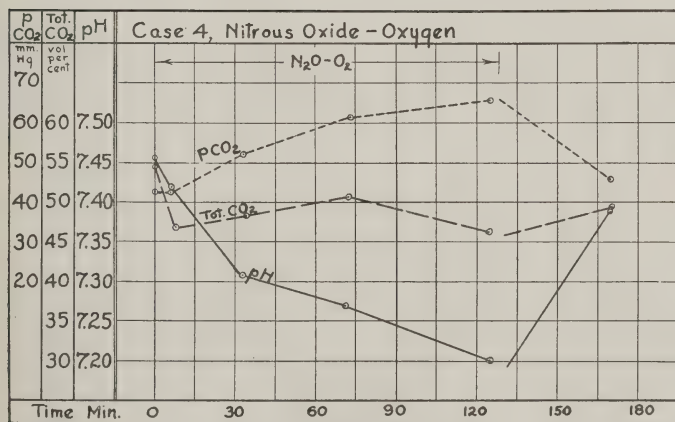


FIG. 6.

TABLE IV.
Nitrous Oxide Anesthesia.

Case No.	Operation.	Duration of anesthesia.	Time blood was drawn.	pH	Total CO ₂ , vol. per cent	CO ₂ tension, mm. Hg	Remarks.
1	Open reduction, fracture of os calcis.	2 hrs. 40 min. Recovery, 55 min.	2 hrs. 35 min. during. 30 min. after. 1 hr. 20 min. after. 2 hrs. 30 " " 2 days after.	7.15	53.2	82.8	Age, 23. Temp. 98.8°F. Resp. 10-20. Pulse 70-140. Preoperative and postoperative condition, good. No nausea, vomiting, or gas distress.
				7.33	39.6	41.8	
				7.40	43.3	38.4	
				7.41	48.2	41.9	
				7.48	58.3	43.8	
2	Subtotal thyroidectomy for exophthalmic goiter.	1 hr. 5 min. Recovery, 35 min.	2 hrs. before. 4 min. after. 1 hr. after.	7.40	50.0	43.9	Age, 38. Temp. 99.0°F. Resp. 20-32. Pulse 110-160. Preoperative condition, good. Postoperative condition, fair. Nausea and vomiting for 12 hrs., no gas distress.
				7.29	45.0	55.9	
				7.38	46.7	43.8	
3	Spinal fusion, tuberculosis of spine.	1 hr. 20 min. Recovery, 30 min.	45 min. during. 2 " after. 40 " "	7.13*	47.6	76.5	Age, 15. Temp. 98.8°F. Resp. 22-30. Pulse 96-140. Preoperative condition, good. Postoperative condition, fair. No nausea or vomiting, moderate gas distress for 48 hrs. 2 oz. ether given at start.
				7.19	47.3	67.6	
				7.36	46.4	45.2	
4	Open reduction, compound fracture of leg.	2 hrs. 10 min. Recovery, 45 min.	2 hrs. before. 6 min. during. 33 " " 1 hr. 9 min. during. 2 hrs. 4 " " 40 min. after.	7.46	54.2	42.5	Age, 18. Temp. 98.6°F. Resp. 20-28. Pulse 100-124. Preoperative and postoperative condition, good. No nausea, occasional vomiting, and slight gas distress for 36 hrs.
				7.43	47.3	42.2	
				7.31	48.0	52.4	
				7.27	51.0	60.8	
				7.20	47.1	65.8	
				7.38	48.2	45.2	

DISCUSSION.

The results that we have obtained upon man are very similar to those reported by Van Slyke, Austin, and Cullen (4) and their coworkers (6) on dogs. However, in the operative cases reported here, one must consider the effects of operation and method of inducing anesthesia besides that of the anesthesia itself. That ether or nitrous oxide anesthesia produces a marked acidosis in animals has been proved very definitely, and although our results are complicated by operation, it is altogether reasonable to believe that the anesthesia produces similar changes in man. Not only is it probable, but our results tend to show that the trauma, and perhaps sensory stimulation, of operation augments the acidosis. For example, Case 3, Table IV, shows an acidosis more severe during hammering and chiseling of the spine than at a later stage when the operative procedures were less drastic. Then too, besides the specific effects of the anesthesia and the operation, the methods ordinarily used at the Massachusetts General Hospital for administering ether or nitrous oxide are such as to cause a certain amount of rebreathing, thus increasing the CO_2 tension of the blood and lowering the pH still more.

It must be pointed out that all the determinations were done on venous blood. That changes in the peripheral circulation or variation in different tissues may distort the results cannot be denied. On the other hand, the writer believes that the pH of the venous blood is a good criterion of that of the arterial blood unless definite influences are made to bear on the peripheral circulation such as marked chilling.

pH.—Our results are in accordance with the observations of Cullen, Austin, Kornblum, and Robinson (6) in that there is a sudden drop in pH as well as total CO_2 during the induction period of ether anesthesia which is followed by a gradual drop during maintenance. Although the cause of this initial acidosis is not understood, it is probable, as the above mentioned authors pointed out, that it is due to a combination of such factors as exertion, anoxemia, and respiratory disturbances.

In nitrous oxide anesthesia such a high percentage of nitrous oxide may be necessary to obtain rapid induction that the patient may experience definite oxygen want and the resulting hyperpnea may lower the CO_2 tension and cause a temporary increase in pH.

This condition is not always obtained and if present the duration is for a few minutes only. If it persists longer the mechanism of induction is at fault.

Total CO₂.—As already mentioned, there is no marked drop in the total CO₂ content of the blood in the closed cone method of ether administration or with the nitrous oxide machine. Figs. 2 and 6 show the immediate drop of total CO₂ and then a gradual rise during the 1st hour or so with a subsequent fall. The increase in total CO₂ is very probably due to the accumulation of CO₂ in the blood as the result of rebreathing; the later decline, to the accumulation of non-volatile acids in sufficient quantities to overcome the effect of high CO₂ tension.

In the open drop method of ether administration the fall in total CO₂ is more marked, the effect of the non-volatile acids not being counteracted by the increased CO₂ tension.

CO₂ Tension.—In all cases of ether anesthesia the CO₂ tension of the venous blood was increased. When the method necessitated rebreathing, the tension occasionally rose high enough to double the normal value. The CO₂ tension, however, without rebreathing, as in the open drop or ether vapor pump methods, still rose somewhat. Cullen and his coworkers observed this high CO₂ tension in dogs during ether and chloroform anesthesia and attributed this faulty respiration to a depression of the sensitiveness of the respiratory center. As pointed out before, in nitrous oxide anesthesia there may be a temporary decrease in CO₂ tension during the first few minutes of induction.

Respiration.—The difficulty of obtaining respiratory records together with blood samples during an operation is obvious, so only a few determinations of minute volume were made. Some such observations were already made by White (12) and reported from these clinics. Fig. 2 shows the respiratory minute volume of an ether case where simultaneous blood samples were obtained. As White has shown, there is usually a very definite increase in volume respiration during the period of induction. After this increase there is a drop and a more or less constant level maintained, usually somewhat above normal, during the period of maintenance. The volume respiration is, of course, greatly influenced by the degree of rebreathing. In ether anesthesia the "excitement hyperpnea" is not accompanied by excessive elimina-

tion of CO_2 from the blood as Henderson and Haggard (2) and White (12) suggested; more likely it is the response to the sudden drop in the pH and rise in CO_2 tension of the blood or more specifically of the tissues of the respiratory center. (See Fig. 2.)

Period of Recovery.—The recovery from the changes in the acid-base balance is fairly rapid and is usually complete in from 1 to 3 hours. The recovery may be divided into two phases; that from CO_2 excess which is rapid and that from alkali deficit which is more prolonged. It is for this reason that the total CO_2 content of the blood may drop for the first 45 minutes or so and then, as the alkali deficit is replenished, rise to normal again. In certain cases, however, as Case 5, Table I, aspiration of vomitus and pulmonary edema may prolong the recovery time for hours.

Cause of Alkali Deficit.—The formation of non-volatile acids during ether anesthesia has usually been looked upon as the result of the formation of acid metabolites due to incomplete oxidation. There is no positive evidence for this view and Leake, Leake, and Koehler (5) found no changes in the concentration of acetone bodies in the blood of etherized dogs. More recently Stehle and Bourne (13) have confirmed these results and found no evidence of increased lactic acid or acetone bodies. These workers found an increase in the elimination of phosphates in the urine after ether anesthesia and believe the drop in available blood alkali can be accounted for in this manner.

Severity of Acidosis.—Our knowledge of the detrimental effect of various grades of acidosis is as yet too limited to speak with any assurance in the matter. We have recently acquired data showing that an acidosis, obtained by acid-feeding in man, of the severity observed in the average operation may be kept up for days without any apparent harm. Although this may be true in the normal, the acidosis of anesthesia may be quite detrimental in an organism already distressed by a variety of physiological insults.

CO_2 Inhalation.—Although there is no CO_2 deficit at the termination of anesthesia, the sensitiveness of the respiratory center has been reduced so that a high CO_2 tension, as may be brought about by CO_2 inhalation, is necessary in order to obtain an increase in respiratory volume. The value of CO_2 inhalation in obtaining a more rapid postoperative elimination of ether and better oxygenation has been tried out in this clinic and reported by Dr. James C.

White (12). The improvement obtained in certain cases, especially where the respiration has been greatly depressed, seems unquestionable. On the other hand, the marked increase in the acidosis produced, although only temporary, makes the expediency of postoperative CO₂ inhalation as a routine procedure a problem that can be solved only by observation of a large series of cases so treated.

The author wishes to take this opportunity to express his appreciation of the help and encouragement given him by Drs. J. H. Means and E. P. Richardson whose cooperation made the above work possible.

SUMMARY.

Observations on human subjects undergoing operation under ether or nitrous oxide anesthesia as carried on routinely in the surgical clinic of this hospital show a considerable degree of acidosis. During the first few minutes of anesthesia there is a sudden marked drop in pH of the blood after which the decrease is gradual. The sudden increase in hydrogen ion concentration is the cause of the initial hyperpnea.

The acidosis is the summation effect of CO₂ excess and alkali deficit.

The CO₂ excess is the result of inefficient respiration probably caused by decreased sensitiveness of the respiratory center. In the closed cone method or with the nitrous oxide machine the CO₂ excess is further augmented by rebreathing.

The postoperative inhalation of CO₂ causes a marked but temporary increase in the acidosis.

The detrimental effect of the acidosis is discussed, but the knowledge available on this matter is as yet too meager to warrant any definite conclusion.

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EFFECT OF INSULIN AND MUSCLE TISSUE ON GLUCOSE IN VITRO.

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INTRODUCTION.

Whereas no doubt exists as to the excellent therapeutic effect of insulin it is still an open question as to how insulin affects the carbohydrate metabolism. The decrease in the blood sugar which is the main immediate result of injection of insulin may be explained in different ways. Some investigators think that insulin increases the burning of sugar, others consider an increased glycogenesis the most probable explanation, and still others see the main effect of insulin in a diminution of the sugar production in the liver. Too few experiments are as yet at hand to decide this question. An important part of the problem of the effect of insulin on the sugar metabolism is to find out what is the immediate result of the effect of insulin on the sugar molecule and in what manner the effect is brought about.

Glucose shows the well known phenomenon of mutarotation. Immediately after pure glucose is dissolved in water the solution shows a specific rotatory power $[\alpha]_D^{20} = > 105^\circ$. This value decreases gradually after from 6 to 24 hours at room temperature to 52.5° . If the solution is heated or if small amounts of alkali are added, this value is reached at once. This phenomenon is interpreted by the assumption that pure glucose in the dry state and immediately after it is dissolved is present in the α form, the specific rotatory power of which is $> 105^\circ$. In solution a part of the α -glucose is transformed into a form with a lower specific rotatory power, β -glucose. This transformation goes on until an equilibrium is established between the α and β forms of the glucose.

β -Glucose was first isolated by Tanret in 1895.¹ He determined that its specific rotatory power was 22.5° . An aqueous solution of β -glucose shows increasing specific rotatory power until it reaches 52.5° , because it is partly transformed into α -glucose until an equilibrium between α - and β -glucose is established.

In later years the existence of still another form of glucose—a γ form—has been assumed. This γ form has not been isolated so far. Only substitution products of it, such as the methylglucoside, have been prepared. This hypothetical γ -glucose is thought to be chemically highly active, to be unstable, and to have a specific rotatory power of *less* than 22.5° .

In 1920 Hewitt and Pryde showed that glucose in aqueous solution, when in contact with living intestinal mucosa, became changed into a chemically more active form, which they assumed to be the γ form of glucose. They advanced the hypothesis that in normal blood an enzyme is present which transforms ordinary glucose (α , β -glucose) into the γ form, whereas it was lacking in the blood of diabetics.

Clark showed in 1917 that the optical rotation of fluid containing glucose was changed if the fluid was perfused through the pancreas. He therefore assumed that the pancreas contained an enzyme capable of converting ordinary glucose to another form which the body could metabolize.

In 1922 Winter and Smith made a comparative study of the reducing and rotatory power of the blood sugar. They found that in blood of animals and of healthy individuals the rotatory power of the sugar, if compared with the reducing power, was always lower than that of the ordinary α , β mixture and sometimes even lower than that of the β -glucose alone.

No such discrepancy was found in blood sugar prepared from diabetics. From these experiments they drew the conclusion that, in normal individuals, the blood sugar is in the form of γ -glucose, whereas in diabetics it is present as α , β -glucose. They furthermore assumed that the action of insulin is to convert α , β -glucose to γ -glucose, which form they considered the diabetic organism able to metabolize.

Eadie repeated Winter and Smith's experiments and obtained identical results. He, however, did not consider the experiments

¹ Tanret named it γ -glucose.

sufficient proof for the presence of the γ -glucose in the blood of normal individuals.

Hewitt expresses doubt as to the reliability of the procedure used by Winter and Smith in order to obtain blood sugar in a state suitable for polarimetry. Winter and Smith, however, have since found that insulin affected pure solutions of α , β -glucose, changing a certain fraction into γ -glucose. Slosse found that pure solutions of α , β -glucose to which insulin was added showed a decrease in rotatory power compared with the reducing power. The solutions were kept at 37° for 24 hours.

Recently Irvine (1923) in a review has discussed the chemical evidence for the presence of a γ form of glucose. He concludes that even if an unsubstituted γ -glucose has not been isolated, the experiments already at hand point to the possibility that such a form is produced in the body as an intermediary product during the glucose metabolism.

In this paper we shall report a series of experiments which we have made on the action of insulin on glucose under various conditions.

EXPERIMENTAL.

Technique.—In all our experiments we compared the rotatory and reducing power of solutions of glucose before and after the solutions had been subjected to the action of insulin under various conditions.

The rotatory power was determined in a Schmidt-Haensch polarimeter. The light was filtered through a saturated solution of potassium dichromate. The reading scale was divided into Ventzke's degrees. Each time, ten readings were made and the average figure was taken. It was found, in numerous controls, that the greatest variation in ten readings was 0.02° .

The reducing power was determined by the method of Hagedorn and Jensen (1923). Each solution of glucose was diluted so that the sugar content in each analysis was about 0.15 mg. of glucose. The determinations were made in quadruple and the average figure was taken.

Controls showed that, by this procedure, the sugar content could be determined without error. In a series of solutions of glucose of varying strengths, from 1.3 to 10 per cent, in which equilibrium

between α - and β -glucose was present, the concentrations of glucose were as calculated from the figures obtained by determining the rotatory and reducing powers respectively. Even in the weakest solutions the concentrations calculated in the two ways always showed differences smaller than 1 per cent of their value.

The insulin used was the Danish preparation of insulin, "Medicinalco" (also called "diasulin"). Ordinarily it contains traces of lactose. The insulin therefore had to be specially prepared.² It contained no lactose and showed in solutions neither reducing nor rotatory power. All our experiments were performed at a temperature of 37°C.

Whereas the mixture of pure sugar solution and insulin was clear and suitable for polarimetry, this was not the case in our later experiments when blood or muscle tissue was added. It, therefore, soon became necessary to devise a special procedure in order to obtain a clear solution of glucose.

The method used by Winter and Smith is very elaborate. It takes 6 to 8 hours, and criticism has been brought forward (Hewitt) as to its reliability. We decided to use dialysis through collodion tubes, dried in 80 per cent alcohol. These tubes are impermeable to proteins but relatively easily permeable to sugar.

Our experiments with blood and muscle tissue differed somewhat with respect to technique. With blood we proceeded as follows: Into a collodion tube we introduced about 40 cc. of a 0.9 per cent solution of sodium chloride. The solution had a temperature of 37°. To this, glucose in varying concentrations, insulin, and a little sodium citrate were added. Then from 4 to 20 cc. of blood from normal individuals were allowed to flow directly into the tube, which was placed in a suitable beaker containing about 40 cc. of a 0.9 per cent solution of sodium chloride. After varying lengths of time samples of the clear dialysate in the beaker were analyzed.

In our later work with muscle tissue the first part of the experiments was performed in bottles. Into a 300 cc. bottle containing 0.9 per cent sodium chloride solution, glucose, insulin, and muscle tissue were introduced. The bottle was rotated continuously in an incubator at 37°. At various intervals samples of about 30 cc. were withdrawn, placed in collodion tubes, and dialyzed at room temperature. After 1½ hours' dialysis, samples of the fluid in the beaker around the tube were analyzed. The muscle tissue used was from guinea pigs and mice. The animals were decapitated, and the muscles were immediately excised, cut into small pieces, and placed in the bottle with the solution. All our experiments were performed at 37°C.

² For this we are indebted to cand. pharm. Gad-Andresen, the chief of the Insulin Laboratory of the Medicinalco Chemical Co. of Copenhagen.

RESULTS.

1. Effect of Insulin on Solutions of Glucose.

A considerable number of experiments were made. The concentration of glucose in the solution varied from 0.3 to 10 per cent. The amount of insulin added varied from 0.2 to 50 Toronto units per 10 cc. of the solution of glucose. The length of time for the experiments ranged from $\frac{1}{2}$ to 72 hours.

All the experiments showed similar results. The values of the concentration of glucose in the samples calculated from the reducing and rotatory power never deviated more than 1 per cent. There was, therefore, no evidence that insulin is able to change α , β -glucose in pure solution into a form with lower specific rotatory power.

2. Effect of Insulin on Glucose When Blood Was Added.

A series of control experiments on solutions of sugar, salt, and blood alone was first made in order to determine whether or not the blood in itself contained substances which might influence the results. Such substances could be γ -glucose which, according to Winter and Smith, is present in normal blood, or they could be uric acid or creatinine which have a reducing but no rotatory power.

In none of a number of such control experiments did the values for the concentration of the glucose calculated on the basis of reducing and rotatory power deviate more than 1 per cent. Similar results were previously reported by Cooper and Walker.

In the experiments in which insulin was added the experimental conditions varied as follows: The glucose concentration ranged from 0.5 to 5 per cent. The amount of insulin added varied from 0.2 to 30 Toronto units per 10 cc. of the solution; the amount of blood, from 10 to 50 per cent of the solution to which it was added. The duration of the experiments ranged from 1 to 24 hours.

In none of the experiments did the concentration of sugar calculated on the basis of the reducing and rotatory power respectively vary more than 1 per cent. *The results of Winter and Smith and of Slosse could therefore not be corroborated either for pure glucose solution or for glucose plus blood.*

3. Effect of Insulin on Glucose When Muscle Tissue Was Added.

In a series of control experiments made on a mixture of glucose and muscle without insulin, no effect was observed. When insulin was added to a glucose-sodium chloride solution containing muscle tissue, a more or less marked change was found: in all the experiments the rotatory power was smaller than the reducing power when determinations were made on the fluid dialyzed through collodion tubes at room temperature for $1\frac{1}{2}$ hours.

Determinations of the content of glucose (by means of the reducing power) were made at the beginning, during, and at the end of the experiments. No change was found. It could therefore be excluded that the sugar was affected in any other way (for instance burned or transformed into glycogen) than changed to another optical form with the same reducing and a lower rotatory power. A further proof of this explanation was the fact that when such a solution showing a discrepancy between reducing and rotatory power was allowed to stand, it steadily increased in its rotatory power until this again, after a certain lapse of time, became equivalent to the reducing power. This difference between the reducing and the rotatory power of a given solution to which insulin and muscle had been added varied considerably in our experiments. We undertook, therefore, to investigate the quantitative effect of a number of factors which evidently were responsible for the variations in our results.

A. Influence of Time on the Transformation of α , β -Glucose.

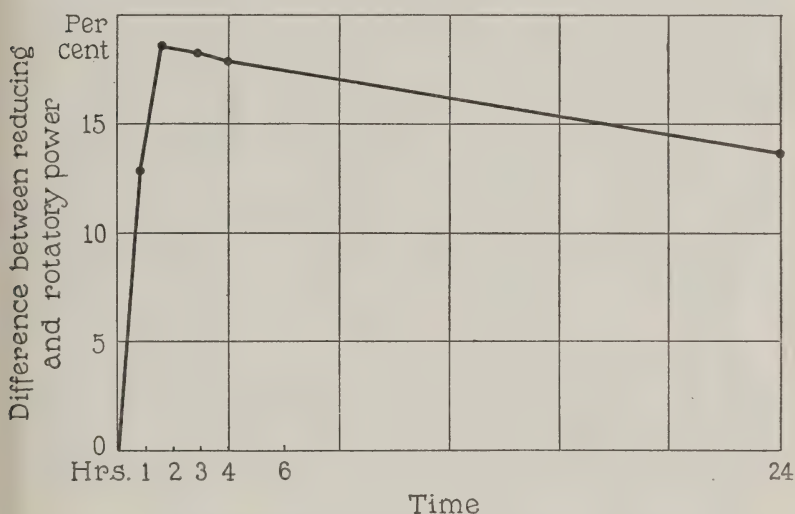
In Table I and in Fig. 1 an experiment is reported in which samples were analyzed after different periods of time. All other conditions were constant. From the results it is seen that the greatest difference between reducing and rotatory power is found after 2 hours. From 3 to 24 hours after the beginning of the experiment this difference again decreases. The reason for this is undoubtedly that the transformation lasts for only 2 hours. After that time the process reverts in the direction of establishing the previous normal equilibrium between the α -glucose and the β -glucose.

In a series of similar experiments with other concentrations of glucose, identical results were obtained. The concentration of transformed glucose was in all experiments at its maximum after 2 or 3 hours. After that time the concentration became lower again.

TABLE I.

Influence of Time on the Transformation of α , β -Glucose.

Time after the beginning of the experiment.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.
hrs.	per cent
1	12.9
2	18.5
3	18.2
4	17.9
6	17.1
24	13.7

FIG. 1. Influence of time on the transformation of α , β -glucose.*B. Which Factors Determine the Cessation of the Process?*

Several possibilities suggest themselves as responsible for the fact that the transformation of the α , β -glucose comes to a standstill after about 2 hours. Our first thought that it was a change in the reaction of the solution was easily excluded. We then proceeded to ascertain whether the insulin or the active substance (or principle) in the muscular tissue was used up or destroyed in the course of the first 2 hours of the experiment.

TABLE II.

Influence of Repeated Additions of Insulin or Muscle on the Transformation of α , β -Glucose.

Time after the beginning of the experiment.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.	Remarks.
<i>hrs.</i>	<i>per cent</i>	
2	9.9	
3	10.5	After the 3rd hr. 50 units of insulin were added.
5	9.5	After the 5th hr. 15 gm. of muscle were added.
7	18.5	
8	17.8	
20	15.1	

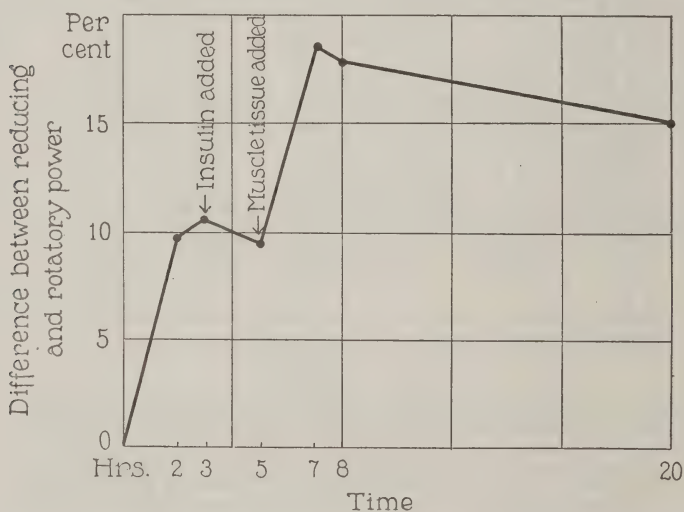


FIG. 2. Influence of repeated additions of insulin and muscle tissue on the transformation of α , β -glucose.

In Table II and Fig. 2 are given the results of an experiment undertaken to throw light on this question. 200 cc. of a 2 per cent solution of glucose in a 0.9 per cent solution of sodium chloride were introduced into the bottle. 15 gm. of muscle tissue and 50 units of insulin were added at the beginning of the experiment.

In samples withdrawn after 2 and 3 hours the difference between the reducing and rotatory power was found to be 9.9 and 10.5 per cent of the reducing value respectively.³ Then 50 more units of insulin were added. This, however, did not increase the transformation of glucose, because in a sample withdrawn after 5 hours the difference was only 9.5 per cent. 15 gm. of muscle were now added. 2 hours later a fourth sample was withdrawn and dialyzed. In this sample the difference had risen to 18.5 per cent. 1 hour later the difference had decreased to 17.8 per cent, showing that the effect of the muscle tissue had lasted for only 2 hours. 20 hours after the beginning of the experiment the difference had gone down to 15.1 per cent.

The experiment shows clearly that the cessation of the process of transformation of the α , β -glucose after 2 hours is not caused by lack of insulin but by lack of muscle tissue. It shows, furthermore, that enough insulin was added at the beginning of the experiment and that the action of the insulin lasts more than 5 hours.

The fact that the process stopped after 2 hours may be explained either by assuming that the 15 gm. of muscle added can transform only a certain quantity of α , β -glucose or by assuming that the active principle (or substance) in the muscles can persist for only 2 hours. In order to decide between these two possibilities the following experiment was made.

15 gm. of muscle tissue were kept in a 0.9 per cent solution of sodium chloride at a constant temperature of 37° for 2 hours, after which glucose and insulin were added. After 2 and 3 hours, samples were taken and dialyzed. No difference was found between the rotatory and reducing value of the dialysate.

This experiment shows that the muscle tissue loses its activity in 2 hours under these conditions. It is therefore necessary to use the muscles immediately after the killing of the animals and to prepare and hash the muscles as quickly as possible.

C. Influence of the Concentration of Glucose on Its Transformation.

The results of five experiments are given in Table III. The concentration of glucose varied from 0.92 to 5.40 per cent. All the other experimental conditions were kept constant. The amount of solution in the bottle was 200 cc. and contained 0.9 per cent sodium chloride. 15 gm. of

³ All the differences are in percentage of the highest; *i.e.*, the reducing value.

muscle tissue and 50 units of insulin were added in each experiment. The duration of the experiment proper was 2 hours (at 37°), and of the dialysis, 1½ hours (at room temperature).

The figures in Table III show that after 2 hours the difference between the reducing and rotatory power is much higher (18.5 per cent) in the experiments with the lowest concentration of glucose than in those with the highest concentration (4.2 per cent).

Two possibilities suggest themselves as a cause of the decrease in the transformation of α , β -glucose with increasing concentration of glucose. One is that the higher concentration in itself in some way prevents the transformation, another is that only a certain quantity of α , β -glucose can be transformed under the conditions given. In order to decide between these two possibilities the following experiment was made.

TABLE III.
Influence of the Concentration of Glucose on Its Transformation.

Concentration of glucose in the bottle.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.
<i>per cent</i>	<i>per cent</i>
0.92	18.5
1.40	13.2
1.66	11.3
2.00	9.9
5.40	4.2

Instead of using 200 cc. of a 2 per cent solution of glucose as in Experiment 4 in Table III, only 100 cc. were used. All the other conditions were the same in the two experiments. After 2 hours the rotatory power of the solution had decreased 19 per cent which is almost double the decrease found in the rotatory power in Experiment 4, Table III, where the concentration of glucose was the same (2 per cent), although the total amount of sugar was twice as large. It seems, therefore, justifiable to state that—within the conditions used in our experiments—the concentration of glucose is of no importance for the transformation and that only a certain quantity of glucose can be transformed.

D. Influence of Amount of Muscle Tissue Added on the Transformation of Glucose.

In Table IV and Fig. 3 there is reported a series of experiments on the relation between the transformation of glucose and the quantity of muscle tissue used. Apart from the variations in the amount of muscle tissue added, all the experimental conditions were kept constant. In the bottle at

the beginning there were 200 cc. of a solution containing 0.9 per cent sodium chloride and 2 per cent glucose. 50 units of insulin were added. The duration of all the experiments was 2 hours at 37° for the experiment proper and 1½ hours at room temperature for the dialysis.

TABLE IV.

Influence of Amount of Muscle Tissue Added on the Transformation of Glucose.

Muscle tissue added.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.	Difference in glucose equivalents between reducing and rotatory powers of dialysate per 5 gm. of muscle tissue added.
<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
5	4.1	4.1
10	7.2	3.6
15	9.9	3.3
20	11.7	2.93
50	16.9	1.69

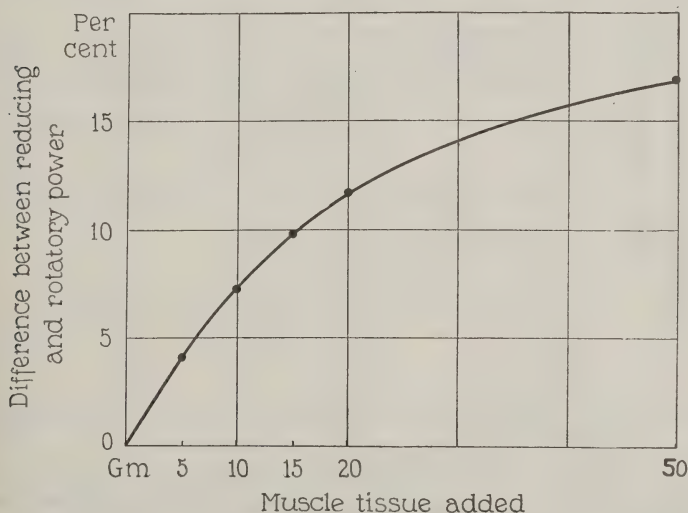


FIG. 3. Influence of amount of muscle tissue added on the transformation of glucose.

The results show that the amount of glucose transformed increases with increasing quantity of muscle tissue added. This increase is, however, not strictly proportional to the increase in the amount of muscle tissue, since relatively more glucose is trans-

formed in the experiments with the smallest amount of muscle tissue, as shown in Fig. 3.

TABLE V.

Influence of Amount of Insulin Added on the Transformation of α , β -Glucose

Amount of insulin added.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.
<i>units</i>	<i>per cent</i>
50	9.9
20	9.9
10	10.5
5	9.3
2	4.9

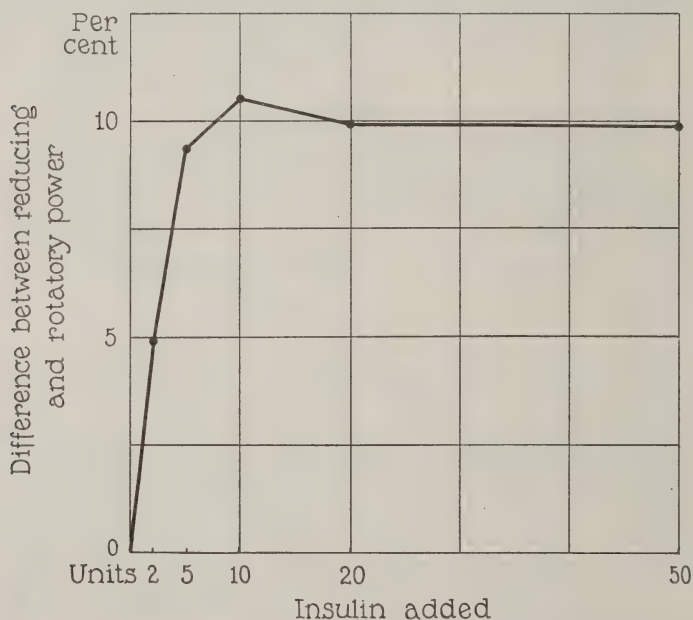


FIG. 4. Influence of amount of insulin added on the transformation of α , β -glucose.

E. Influence of Amount of Insulin Added on the Transformation of α , β -Glucose.

In a series of experiments given in Table V and Fig. 4 the amount of insulin added varied from 50 to 2 units. The other experimental conditions were kept constant and were as follows: 200 cc. of 0.9 per cent solution of

sodium chloride with 2 per cent glucose. 15 gm. of muscle tissue were used in each experiment. The duration was 2 hours at 37° for the experiment proper and 1½ hours at room temperature for the dialysis.

The results show that 10 or possibly 5 units of insulin suffice to produce a maximum effect under the experimental conditions used. This suggests that the first part of the curve in Fig. 4 could be used as a means of standardizing insulin. Experiments are in progress to study this point.

F. Influence of Temperature on the Process.

An experiment was performed at 20°. Apart from the temperature, the experimental conditions were as usual. After 2 hours no decrease was observed in the rotatory power of the solution. Then the temperature was raised to 37° and 15 gm. of muscle tissue were again added. After 2 hours the usual decrease in the rotatory power was found, whereas the reducing power remained constant as usual.

TABLE VI.

Experiments to Show How Far the Transformation of Glucose Can Be Pushed by Repeated Additions of Muscle Tissue and Insulin.

Time after the beginning of the experiment.	Difference in glucose equivalents between reducing and rotatory powers of dialysate.	Remarks.
hrs.	per cent	
2	13.2	Every other hr. 15 gm. of muscle and 10 units of insulin were added.
4	24.2	
6	34.2	
8	43.8	
10	43.6	

G. How Far Can the Transformation of Glucose Be Pushed?

This problem is of considerable theoretical as well as practical importance. In Table VI an experiment is reported in which it was possible by repeated addition of muscle tissue and insulin to increase the transformation considerably. The experimental conditions were the following.

When the experiment was started, 2.8 gm. of glucose were added to 200 cc. of a solution of 0.9 per cent sodium chloride. The concentration of glucose was, therefore, at the beginning 1.4 per cent. Then 10 units of insulin and 15 gm. of muscle tissue were added. The bottle was rotated

continuously at 37°. After 2 hours a sample of 20 cc. was withdrawn for dialysis. 10 units of insulin and 15 gm. of muscle were again added. After the 4th hour, the second sample was withdrawn, etc. In this way five samples were withdrawn in 10 hours.

The samples were subjected to dialysis for 1½ hours at room temperature. Then the difference between the reducing and rotatory power was determined. The figures in Table VI show that more and more glucose is transformed during the experiment. After the 8th hour the difference between the reducing and rotatory power is 43.8 per cent (of the reducing value) which corresponds to a specific rotatory power of 29.5°. After 10 hours the same value was found as after the 8th hour, and the experiment was stopped.

Whether the transformation is a formation of γ -glucose or simply a change in the equilibrium between the α and the β form at the cost of the concentration of the α form we are—as previously mentioned—as yet unable to say. However, in either case it appears that not all the glucose is transformed because the specific rotatory power of the solution does not decrease below 22.5°. This is most likely due to the reversibility of the process, which goes back during the dialysis.

H. Influence of Gastric Juice on the Speed of the Reverse Process.

In one experiment normal gastric juice was added to a solution containing a certain amount of transformed glucose. It was found that this did not increase the rate of the reverse process.

DISCUSSION.

The results obtained in these experiments seem to be of importance for our understanding of the carbohydrate metabolism. They lend support to the theory that the first step in the carbohydrate metabolism is a transformation of the ordinary α , β -glucose (equilibrium glucose, Tanret's β -glucose) into a form with a lower specific rotatory power but with the same reducing power. This transformation is produced by means of insulin and a principle or substance present in living muscle tissue. Insulin alone or this substance alone cannot produce this effect. Whether or not other tissues possess the same property we do not yet know.

On first consideration our results might seem to support the theory of Winter and Smith regarding the existence of γ -glucose in the blood of normal individuals. However, in none of our experiments did the specific rotatory power of the sugar solutions decrease below 22.5° .

Our results may therefore be explained either by assuming a formation of γ -glucose or simply by a change in the equilibrium between the α - and β -glucose in such a way that the concentration of β -glucose is increased at the cost of the α form. According to either view the decrease in the rotatory power must go parallel with the amount of glucose transformed. The observed difference between the observed reduction power and rotatory power must, therefore, be a quantitative expression of the amount of glucose transformed by the combined action of insulin + muscle tissue under the conditions given.

SUMMARY.

1. A procedure is described for obtaining a clear sugar-containing fluid from mixtures of glucose with insulin, blood, or muscle tissue. The method consists in dialyzing through collodion tubes, dried in 80 per cent alcohol.

2. When insulin was added to solutions with or without blood from normal individuals, no change took place in the rotatory power of the dialysate. This suggests that if the blood sugar in normal individuals is changed from the ordinary form (α , β -glucose) to a form with less optical rotatory power, the change probably takes place extravascularly.

3. When insulin and muscle tissue were added to solutions of ordinary glucose (α , β -glucose, equilibrium glucose, Tanret's β -glucose), a decrease in the optical rotatory power of the dialysate took place without any change in the reducing power. Insulin alone, or muscle tissue alone, could not produce this change.

According to our results this change may be due either to a change in the relative amount of α - and β -glucose in the mixture with formation of β -glucose at the cost of α -glucose, or it may be due to formation of a variety of glucose with a specific rotatory power lower than that of the β -glucose (perhaps γ -glucose). This transformation cannot be produced by insulin alone or by muscle tissue alone.

4. The influence on this process of a number of factors (concentration of glucose, amount of insulin, amount of muscle tissue, temperature) was studied quantitatively.

5. It was found that muscle tissue was active for only 2 hours. Muscles kept for 2 hours at 37° in 0.9 per cent solution of sodium chloride were inactive. The active principle or substance in the muscles is, therefore, presumably connected with vital processes in the cells.

6. Under the conditions of these experiments the process by which glucose is transformed into a form with lower rotatory power is reversible. The reverse process is, however, relatively slow. For this reason absorption and utilization of transformed glucose from the intestines may be possible. This question is being studied further.

7. Addition of normal gastric juice to a dialysate containing transformed glucose did not increase the speed of the reverse process.

8. In none of our experiments did we find any evidence that insulin (alone or with blood or muscle tissue) is able either to burn sugar or to transform it into glycogen.

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INACTIVATING ACTION OF SOME FATS ON VITAMIN A IN OTHER FATS.

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According to Drummond's investigations (1919), hydrogenated whale oil does not contain fat-soluble A, although the raw whale oil is rich in this vitamin. This result was corroborated by experiments conducted in our laboratory. But in one of them an unexpected phenomenon was observed. Experimental animals, receiving a basal food mixture, not containing vitamin A, mixed with 10 per cent hydrogenated whale oil, did not grow as usual, and control animals, receiving the basal food mixture mixed with 10 per cent butter fat, grew normally. Other control animals, receiving the basal food mixture with 10 per cent butter fat and 10 per cent hydrogenated whale oil, did not grow, but behaved like animals starved for vitamin A, although the food of these animals contained a sufficient amount of vitamin A-containing butter fat in addition to the hydrogenated whale oil.

In experiments by McCollum, Simmonds, and Pitz (1916), on the toxic effect on rats of certain vegetable oils, the hydrogenated whale oil was considered to be toxic. This assumption did not agree with the results of Erlandsen and his coworkers (1917), who found no toxic effects when feeding adult human beings, through protracted periods, food mixtures containing hydrogenated whale oil. Possibly young animals behave differently than adult human beings. This was the starting point of the experiments on rats, which resulted in another explanation of the phenomenon observed.

EXPERIMENTAL.

Basal Food Mixture.—200 gm. of caseinogen, 30 gm. of agar, 50 gm. of autolyzed and dried yeast, 470 gm. of rice-starch, and 50 gm. of salt mixture.

The caseinogen was purified by repeated washing with hot alcohol and with ether, then heated in thin layers to 105°C. for 24 hours (as recommended by Drummond and Coward (1920)). The agar was powdered and boiled in 96 per cent alcohol. The autolyzed yeast was dried at a temperature lower than 40°C. The rice-starch was boiled twice in 96 per cent alcohol. The salt mixture was that recommended in the report of the Medical Research Council.¹ Butter fat was prepared from butter, melted on the water bath, separated from water and casein in a separating funnel, and filtered through cotton.

The rats were kept in a thermostat at 22°C. Each rat was confined in a special cage made of iron wire net. The urine and feces fell through the meshes of the floor. On one side of each cage was an eating-chamber with a food glass. This construction of the cages prevents the rats from eating the feces and from soiling the food. The amount of food eaten was always controlled by weighing the food glass. All rats were pied rats. The experiments were started when the young rats weighed 30 to 50 gm.; in one experiment (No. 2), 50 to 60 gm.

RESULTS.

The first two experiments were made in order to ascertain whether hydrogenated whale oil had a toxic effect on rats. The hydrogenated whale oil used was a Norwegian brand, which had been stored in the laboratory for a rather long time. The fat was white, without taste, but had, on heating, a faint fish oil smell. Melting point, 38.2°; iodine value, 76; saponification value, 194; free fatty acids, 0.06 per cent; caloric value, 9.52 Cal. per gm.

Experiment 1.—7 young rats (5 males, 2 females), weighing 40 to 49 gm. (Fig. 1). Control Rats A 1 and A 2 received 90 per cent basal food mixture + 10 per cent butter fat; Control Rat A 3, 80 per cent basal food mixture + 20 per cent butter fat. Experimental Rats B 1 and B 2 received 80 per cent basal food mixture + 20 per cent hydrogenated whale oil.

Experimental Rats C 1 and C 2 received 80 per cent basal food mixture + 10 per cent butter fat + 10 per cent hydrogenated whale oil.

The graphs of Fig. 1 show that Control Rats A 1, A 2, and A 3 grew normally. During the first 4 weeks of the experiment the growth of Rats B 1 and B 2 (receiving hydrogenated whale oil) as

¹ Medical Research Council (1924), p. 14.

well as the growth of Rats C 1 and C 2 (receiving a mixture of butter fat and hydrogenated whale oil) was very slight compared with the growth of the control rats. This agreed with the former

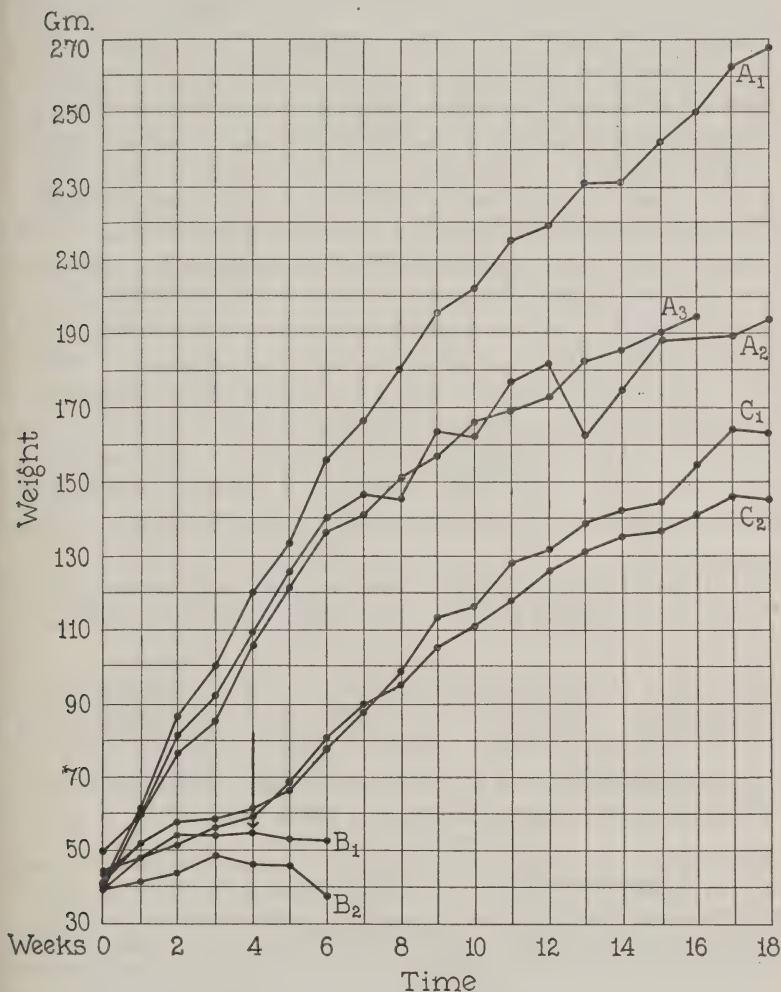


FIG. 1. Experiment 1. Hydrogenated whale oil.

observation. When the food was made up with a mixture of hydrogenated whale oil and butter fat the rats did not grow, although the butter fat of their food contained a sufficient amount of vitamin A.

As mentioned above, the phenomenon may be due to the toxic effect of the hydrogenated whale oil, but another explanation is also possible: the hydrogenated whale oil may have a destructive action on the vitamin A of the butter fat. In order to test the validity of this supposition, the manner of feeding Rats C 1 and C 2 was changed at the beginning of the 5th week of Experiment 1 (indicated by the arrow in Fig. 1). The composition of the diet was not changed. These rats continued to receive 80 per cent basal food mixture + 10 per cent butter fat + 10 per cent hydrogenated whale oil. Formerly the butter fat and the hydrogenated whale oil had been melted at a low temperature, mixed, and stored for daily use in composing the food mixture, but from the 5th week on the butter fat was no longer mixed with the hydrogenated whale oil. An amount of butter fat, corresponding to 10 per cent of the food eaten by these rats, was weighed out daily and fed separately to Rats C 1 and C 2. The effect produced by this change in the manner of giving the food ingredients was astonishing. After the change, Rats C 1 and C 2 immediately started growing at a normal rate (Fig. 1). Accordingly, the hydrogenated whale oil could not have had any toxic effect on the growth of the rats.

Rats B 1 and B 2, receiving 90 per cent basal food mixture + 10 per cent hydrogenated whale oil, lost weight and died after 6 to 7 weeks.

The result of this experiment points to the conclusion that hydrogenated whale oil has no toxic effect on the growth of young rats, but destroys (or inactivates in some way) the vitamin A of butter fat when these two fats are mixed together after melting.

To test this conclusion, Experiment 2 was made.

Experiment 2.—5 young rats (4 males, 1 female), weighing 50 to 60 gm. (Fig. 2). Control Rat A received 90 per cent basal food mixture + 10 per cent butter fat. Rats B 1, B 2, C 1, and C 2 received the same diet; *viz.*, 80 per cent basal food mixture + 10 per cent hydrogenated whale oil + 10 per cent butter fat. Rats B 1 and B 2 received the butter fat mixed with the hydrogenated whale oil (after melting) and with the basal food ingredients, while Rats C 1 and C 2, on the contrary, received only the hydrogenated whale oil mixed with the basal food mixture and were supplied daily with the stipulated amount of butter fat separately.

The result is shown in Fig. 2. Rats C 1 and C 2, receiving the butter fat separately, grew at a normal rate as did Control Rat A, but Rats B 1 and B 2, receiving the butter fat mixed with the hydrogenated whale oil, declined after 6 weeks and died in the 7th to 8th week of the experiment.

The result of Experiment 2 corroborates the conclusion drawn from Experiment 1 as to the inactivating power of the vitamin A of butter fat by hydrogenated whale oil, when the two fats are mixed after being melted.

The raw whale oil is rich in vitamin A (Drummond, 1918-19 and 1919) and accordingly does not destroy or inactivate this vitamin. The inactivating faculty must have come into existence during the treatment which the raw whale oil has undergone in

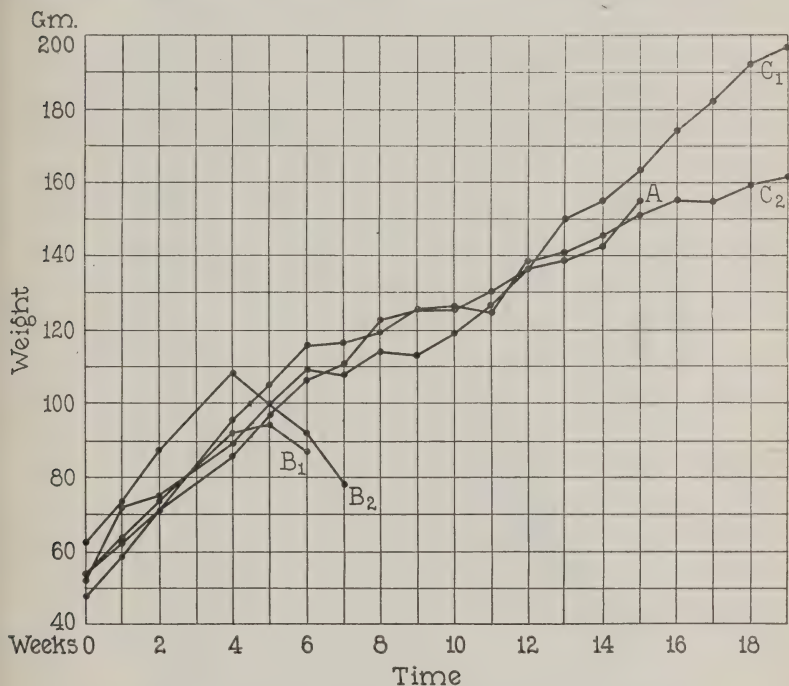


FIG. 2. Experiment 2. Hydrogenated whale oil.

being turned into hydrogenated oil. In consequence it was of interest to test whether other hydrogenated oils besides hydrogenated whale oil inactivated vitamin A.

In Experiments 3, 4, and 5 the action of two hydrogenated and refined vegetable oils (coconut oil and hemp-seed oil) on the vitamin A of butter fat was examined and compared with the action of a non-hydrogenated but refined vegetable oil (coconut oil). The experiments were carried out in the same way as Experiment 2.

Experiment 3.—6 young rats (4 males, 2 females), weighing 38 to 47 gm. (Fig. 3). Control Rat A received 90 per cent basal food mixture + 10 per cent butter fat. Rats B 1, B 2, C 1, and C 2 received the same diet; *viz.*, 80 per cent basal food mixture + 10 per cent hydrogenated and refined coconut oil + 10 per cent butter fat. Rats B 1 and B 2 received the butter fat mixed with the hydrogenated coconut oil (after melting) and the basal

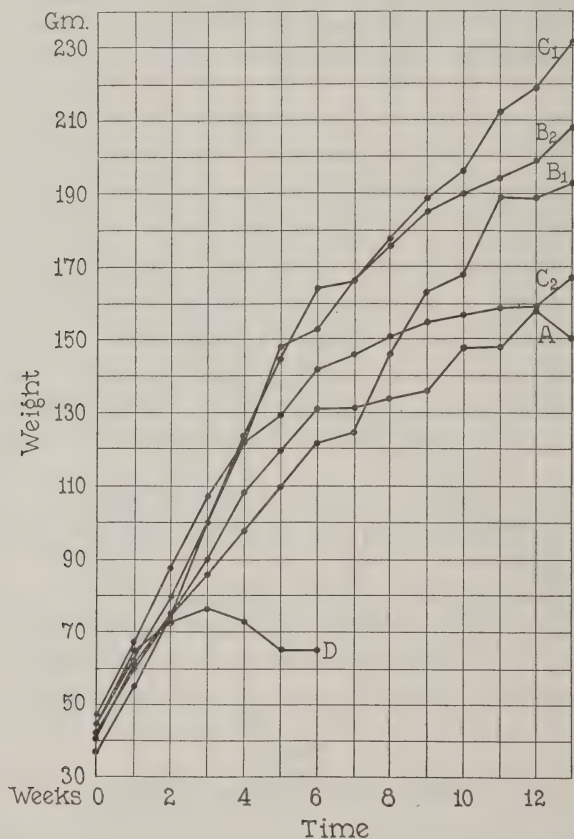


FIG. 3. Experiment 3. Hydrogenated and refined coconut oil.

food ingredients, while Rats C 1 and C 2, on the contrary, received only the hydrogenated coconut oil mixed with the basal food mixture but were supplied daily with the stipulated amount of butter fat separately. The hydrogenated and refined coconut oil had a melting point of 30.5°C. and an iodine value of 3.6. Rat D received 80 per cent basal food mixture + 20 per cent hydrogenated coconut oil.

Experiment 4.—5 young rats (3 males, 2 females), weighing 30 to 50 gm. (Fig. 4). Rats B 1, B 2, C 1, and C 2 received 80 per cent basal food mixture + 10 per cent refined but non-hydrogenated coconut oil + 10 per cent butter fat. Rats B 1 and B 2 received the butter fat mixed with the coconut oil (after melting) and the basal food mixture, while Rats C 1 and C 2 re-

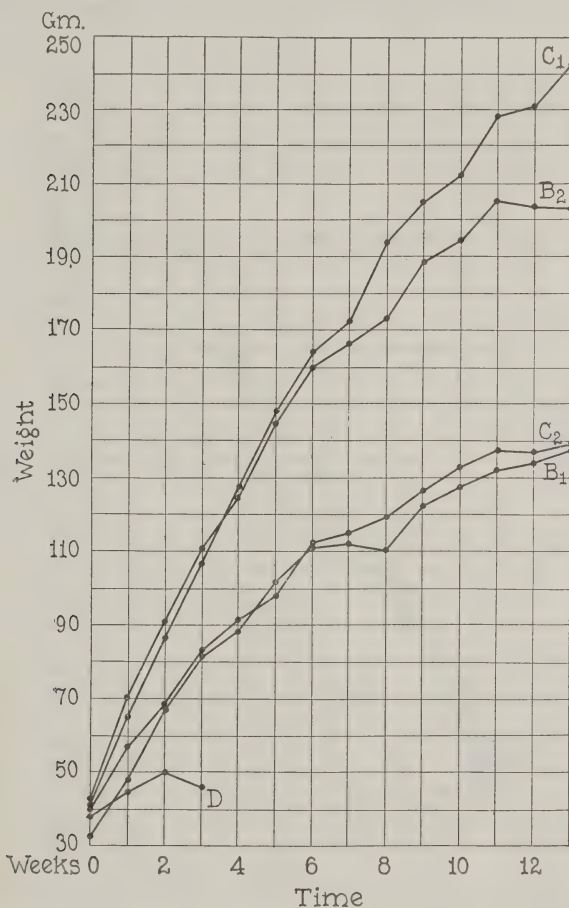


FIG. 4. Experiment 4. Refined but non-hydrogenated coconut oil.

ceived the butter fat separately. The refined coconut oil had a melting point of 25.0°C. and an iodine value of 8.8. Rat D received 80 per cent basal food mixture + 20 per cent non-hydrogenated coconut oil.

Experiment 5.—7 young rats (5 males, 2 females), weighing 30 to 50 gm. (Fig. 5). Control Rat A 1 received 80 per cent basal food mixture + 20 per cent butter fat; No. A 2, 90 per cent basal food mixture + 10 per cent butter

fat. Rats B 1, B 2, C 1, and C 2 received 80 per cent basal food mixture + 10 per cent hydrogenated hemp-seed oil + 10 per cent butter fat. Nos. B 1 and B 2 received the butter fat mixed with the hemp-seed oil (after melting) and the basal food mixture. Nos. C 1 and C 2 received the butter fat separately. The hydrogenated hemp-seed oil had a melting point of 35°C. Rat D received 80 per cent basal food mixture + 20 per cent hydrogenated hemp-seed oil.

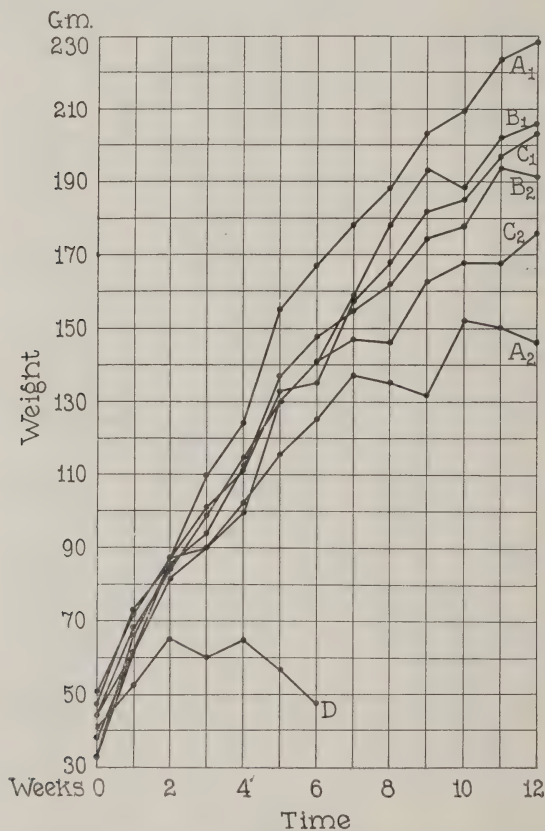


FIG. 5. Experiment 5. Hydrogenated hemp-seed oil.

The results of these three experiments are shown in Figs. 3, 4, and 5. The graphs of the D rats in the three figures show that vitamin A is not present in traceable amounts either in the refined or in the hydrogenated coconut oil or in the hydrogenated hemp-seed oil.

The graphs of the B and C rats in the three experiments rise normally, similar to those of the A rats; *i.e.*, the growth of all B rats has been normal as well as the growth of all C rats. Accordingly, these three vegetable fats had no toxic effect on the growth of the rats and the mixing of the three fats with butter fat had no inactivating effect on the vitamin A of the butter fat.

Thus far the inactivating action on vitamin A has only been found in one fat; *i.e.*, in the hydrogenated whale oil, but not in hydrogenated vegetable oils. The process of hydrogenating does not produce the inactivating faculty, or at any rate does not produce it in all fats.

In the next experiment, lard was examined. The experiment was carried out in the same manner as the preceding ones.

Experiment 6.—6 young rats (4 males, 2 females), weighing 40 to 55 gm. (Fig. 6). Control Rat A 1 received 80 per cent basal food mixture + 20 per cent butter fat; Control Rat A 2, 90 per cent basal food mixture + 10 per cent butter fat. Rats B 1, B 2, C 1, and C 2 received 80 per cent basal food mixture + 10 per cent lard + 10 per cent butter fat. Nos. B 1 and B 2 received the butter fat mixed with the lard (after melting) and the basal food mixture, while Nos. C 1 and C 2 received the butter fat separately.

The graphs in Fig. 6 show the unexpected result that while the C rats grew normally, as did the controls (Nos. A 1 and A 2), the B rats stopped growing after 3 weeks and died in the 8th week of the experiment. The growth of the C rats shows that lard has no toxic effect on the rats. No other difference existed between the diet of the B rats and that of the C rats than that the B rats received their butter fat mixed with lard (and the basal mixture), while the C rats received their butter fat separately. The result points to the conclusion that the lard used in this experiment did inactivate the vitamin A of butter fat when the two fats were mixed after melting.

Lard has been extensively used in experiments on vitamins, and the question about presence or absence of vitamin A in lard has been much discussed and examined. But the question arising from the result of Experiment 6 is different.

The lard used in this experiment inactivated the vitamin A of butter fat. Not every sort of lard possesses this inactivating power. Osborne and Mendel (1913) have published experiments in which rats grew normally, although the diet contained a mixture

of butter fat and lard. The question is about the origin and treatment of the lard used in Experiment 6.

The lard was a brand of "guaranteed genuine Danish lard" from a slaughter-house in Jutland. Probably the lard did not contain foreign fats, nor had it undergone any intensive manufacturing processes. But the origin and preparation of this lard had not been submitted to any control on the part of the labora-

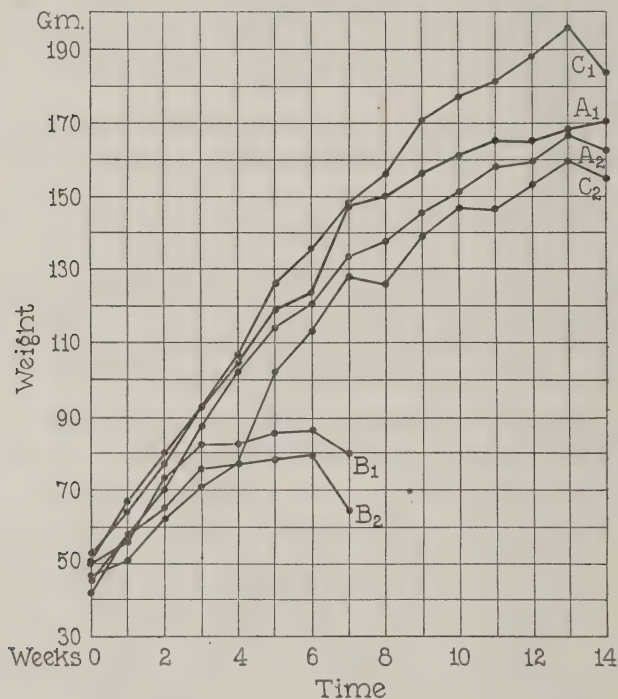


FIG. 6. Experiment 6. Lard.

tory. In the laboratory the lard had been melted and filtered through cotton, then heated in thin layers to 102–105°C. for 24 hours, exposed to the air. The purpose of this treatment was to free the lard from any traces of vitamin A (Drummond and Coward, 1920). This aeration of the melted lard at high temperature might possibly have originated the vitamin A-destroying faculty by generating peroxides. Peroxides are said to be present in "blown oils" prepared by blowing air through the hot oils.

The problem was studied by examining (1) whether raw and untreated abdominal fat from a pig inactivated the vitamin A of butter fat, or (2) whether the fat acquired the inactivating faculty through being heated in the manner described. Experiments 7 and 8 deal with these questions. Experiment 7 deals with the raw fat; Experiment 8, with the heated. The abdominal fat from a pig was brought to the laboratory immediately after slaughter. The experiments with the raw fat and with the heated fat were started simultaneously.

Experiment 7.—The abdominal fat from the pig was melted in a funnel at a temperature below 50°C. (about 40–50°C.) and filtered through several layers of gauze. The cooled and stiffened fat was not further treated. 7 young rats (3 males, 4 females), weighing 33 to 42 gm., were used. Control Rat A 1 received 90 per cent basal food mixture + 10 per cent butter fat. Rats B 1, B 2, C 1, and C 2 received the same diet, but Nos. B 1 and B 2 received butter fat mixed with the raw pig fat, while Nos. C 1 and C 2 received the butter fat mixed with the basal food mixture and were served the raw pig fat separately. Rats B 1 and B 2 received the basal food mixture, and each rat received in addition daily a mixture of 1 gm. of butter fat and 1 gm. of raw pig fat. The butter fat and the pig fat were mixed after melting, and stored. A sufficient amount for several weeks was prepared at one time. A new supply had to be prepared three times during the experiment. Rats C 1 and C 2 received 90 per cent basal food mixture + 10 per cent butter fat, and each rat received in addition daily 1 gm. of raw pig fat, served separately. Rats D 1 and D 2 received 90 per cent basal food mixture + 10 per cent raw pig fat.

The result of Experiment 7 is seen in Fig. 7. The graphs of Rats D 1 and D 2 show a deficiency of vitamin A in the raw pig fat. Comparing the graphs of Rats B 1 and B 2 with the graphs of Rats C 1 and C 2 (and with the graphs of Control Rat A 1), it is evident that the raw, untreated pig fat does not delay the growth of the rats and does not inactivate the vitamin A of butter fat when the two fats are mixed after melting.

Experiment 8.—Started at the same time as Experiment 7 and with a part of the same pig fat. At the beginning of the experiment all the pig fat to be used was heated in thin layers to 102–105°C. for 24 hours, exposed to the air. 4 young rats (2 males, 2 females), weighing 36 to 43 gm., were used. All the rats received the same diet. Rats B 1 and B 2 received the basal food mixture, and each rat received in addition daily a mixture of 1 gm. of butter fat and 1 gm. of heated and aerated pig fat. Mixture, storage, and preparation were the same as in Experiment 7. Rats C 1 and C 2 received 90 per cent basal food mixture + 10 per cent butter fat, and each rat received in addition daily 1 gm. of heated pig fat, served separately.

Fig. 8 shows the result of Experiment 8. Rats C 1 and C 2, receiving the butter fat and the heated pig fat separately, grew normally. The heated pig fat has no toxic effect on the growth of the rats. Rats B 1 and B 2, receiving the butter fat mixed with the heated pig fat (after melting at about $50^{\circ}\text{C}.$), stopped growing after 9 to 11 weeks and died after 12 to 13 weeks. It may be concluded

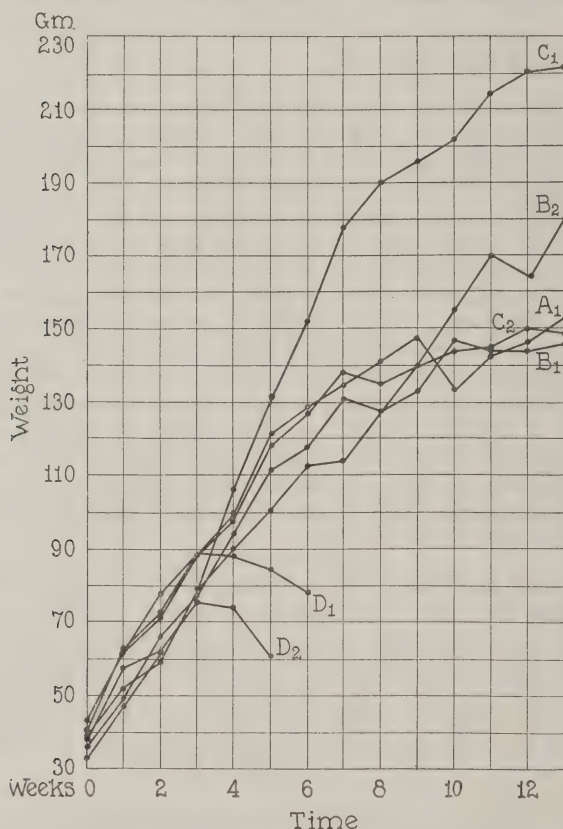


FIG. 7. Experiment 7. Abdominal fat from a pig.

from the results of Experiments 7 and 8 that the raw abdominal fat from a pig is without effect on the vitamin A of butter fat, when the two fats are mixed after melting. After being heated for 24 hours to $102-105^{\circ}\text{C}.$ on exposure to the air, the same pig fat acquires the faculty of inactivating the vitamin A of butter fat when the two fats are mixed after melting.

Rats B 1 and B 2 in Experiment 8 stopped growing later than the rats receiving a diet devoid of vitamin A usually do (see the D rats in other experiments). This suggests that not all the vitamin A of the butter fat had been inactivated by the heated pig fat in Experiment 8.

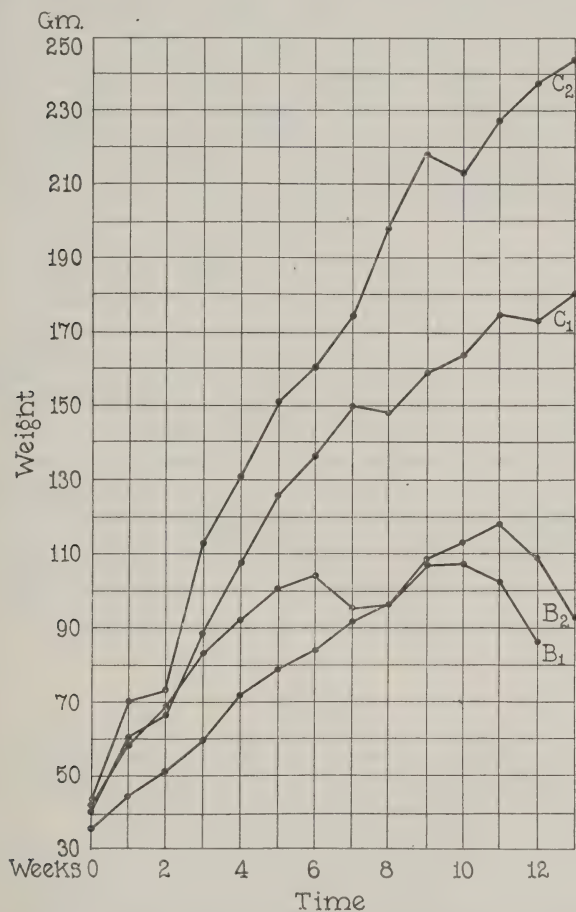


FIG. 8. Experiment 8. Heated abdominal fat from a pig.

DISCUSSION.

The vitamin A-inactivating power of the lard is evidently due to heating the fat on exposure to the air. As mentioned before,

peroxides are possibly generated in the lard by this treatment. If so, the oxidizing action of the peroxides would explain the destruction of vitamin A.

The inactivating action on the vitamin A in butter fat of the brand of hydrogenated whale oil in Experiments 1 and 2 has probably to be explained in some other way than by a similar action in lard. The whale oil being hydrogenated, it is difficult to consider it an oxidizing agent. Certainly it must be granted that the hydrogenated whale oil used had been stored for some years in the laboratory and the possibility of peroxides having been generated in it cannot strictly be excluded. Experiments with recently prepared hydrogenated whale oil will have to be made.

In the literature on vitamins the inability of rats to grow on some food mixtures in which the vitamin A-containing substance was mixed with some fats has been explained by supposing a toxic action of these fats. The possibility of these results being dependent on a vitamin A-destroying action and not on a toxic effect of the fats concerned appears not to be excluded.

In practice the knowledge as to the faculty of some fats to destroy the vitamin A of other fats may be of some consequence in the manufacture of margarine and in cookery.

In the manufacture of margarine, fats containing vitamin A are sometimes mixed with other fats. In cookery, fat, heated and aerated in a frying-pan, is at times mixed with vitamin A-containing substances.

SUMMARY.

1. Rats did not grow on an apparently adequate diet when the butter fat yielding the vitamin A of the diet was mixed (after melting) with a brand of hydrogenated whale oil.

2. Experiments showed that the hydrogenated whale oil had no toxic action on the growth of rats but had an inactivating action on the vitamin A of butter fat when the two fats were mixed after melting at a low temperature.

3. Two hydrogenated vegetable oils (hydrogenated coconut oil and hydrogenated hemp-seed oil) and a non-hydrogenated vegetable oil (coconut oil) showed neither any toxic effect on the growth of rats nor any inactivating action on the vitamin A of butter fat,

this action accordingly not being regularly connected with the process of hydrogenating.

4. Untreated abdominal fat from a pig did not inactivate the vitamin A of butter fat. After being heated in thin layers to 102–105°C. for 24 hours on exposure to the air, the same pig fat acquired an inactivating action on the vitamin A of butter fat when the two fats were mixed after melting. This inactivating action is supposed to depend on the generation of peroxides in the aerated heated fat, the vitamin A of butter fat being destroyed by these peroxides through oxidation.

5. The bearing of the results on the interpretation of previous experiments and on the manufacture of margarine and some problems in cookery is pointed out.

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ON THE SULFURIC ACID REACTION OF BUTTER FAT AND
THE DISAPPEARANCE OF THE REACTION FROM
VITAMIN A-CONTAINING BUTTER FAT
THROUGH THE ACTION OF OXI-
DIZED FAT.

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In 1922 Drummond and Watson published investigations on the sulfuric acid reaction of liver oil. They found that the reaction was not only yielded by cod liver oil but also by liver oil from a number of other animals. They also made comparisons between color indices, found by diluting the liver oils in petroleum ether, and the growth-promoting power of the oils.

They found that a high color index and a strong growth potency were parallel.

Thus there seems to be a certain relation between the growth factor and the chromogenic substance in the liver oil which is the cause of the sulfuric acid reaction. The authors, however, do not wish to identify these two substances, although they present several points of conformity. Both are very sensitive to oxygen and both are thermostable if heated in the absence of air. Drummond and Watson heated to 100°C. a sample of liver oil with an especially high color index and strong growth-promoting power, and passed a current of air through it. At the end of every hour they took a sample, which was tested for color index and growth-promoting potency. These factors decreased parallelly and were equal to zero after 5 hours of heating. The animals which were fed these samples, after being heated for 5 hours or more, developed xerophthalmia, and several of them died from vitamin A deficiency.

Poulssohn and Weidemann (1923) modified the technique of Drummond and Watson. In order to avoid the carbonization caused by the sulfuric acid, which they feared might conceal weak reactions, and to obtain a constant acid concentration, they employed chloroform saturated with sulfuric acid. They found, as did Drummond and Watson, that a high color index and a strong activity, as a rule, correspond.

Drummond and Watson tried the reaction in butter fat and found that it is less potent than in liver oil. This they thought was due to the quantity

of vitamin A being about 250 times less in butter fat than in liver oil (Zilva and Miura, 1921). Poulsson and Wiedemann did not succeed in making butter fat yield a reaction.

I first tried the experiment according to the information given by Poulsson and Wiedemann, but in spite of all my attempts to reproduce their experiment I did not succeed in producing the reaction in any kind of liver oil at my disposal. Guided by the statements of Drummond and Watson, I therefore employed petroleum ether as a means of dilution and pure sulfuric acid, sp. gr. 1.84, as a reagent.

Method.

The experiment is made with a total quantity of 5 cc. First, the petroleum ether needed is measured out with a pipette, then

TABLE I.

Liver oil concentration.	Reaction.	Remarks.
<i>per cent</i>		
2.5	+++	
1.25	+++	
0.63	+	
0.31	0	Solution faintly opalescent.

+++ = strong color reaction; ++ = medium strong reaction; + = faint reaction; and 0 = no reaction.

is added the fatty substance which has, if necessary, been melted in advance by gentle heating on a water bath. From a pipette 1 drop of sulfuric acid is added. The glass is shaken, and the bluish violet-purple color appears quickly and as quickly disappears. After a few minutes a small colored deposit of carbonized organic substance is precipitated.

By this method I examined different samples of liver oil (Table I).

The other two samples of liver oil reacted in the same manner.

Experiments with Butter Fat.

The butter fat is of the same sort as that employed at the Institute as a vitamin A-containing substance for the experiments on

rats. It consists of common butter, heated gently on a water bath and kept in a separatory funnel. The water with the separated casein is then tapped off, and the butter fat strained through cotton.

In 1920 Drummond and his collaborators found that lard contained vitamin A if the pigs were fed on greens. Vitamin A was, however, found only in small quantities. These authors further discovered that lard lost its vitamin content if heated in an open pan in the air. Hopkins (1920) and Drummond and Coward (1920) have proved that this is due to oxidation.

TABLE II.

Butter fat.	Petroleum ether.	Butter fat.	Reaction.
<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	
1.25	3.75	25	++
1.00	4.00	20	++
0.50	4.50	10	+
0.25	4.75	5	+
0.10	4.90	2	0

TABLE III.

Lard.	Petroleum ether.	Lard.	Reaction.
<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	
2.5	2.50	50	0
1.25	3.75	25	0
1.00	4.00	20	0
0.50	4.50	10	0

I therefore tried to make lard yield a reaction. The lard employed was produced from abdominal fat from a pig, melted out at as low a temperature as possible, and then strained through gauze.

At all the concentrations, no color was apparent, only a faint opalescence.

In 1923 Fridericia recorded some experiments on mice in which it was stated that if the mice were fed on butter fat and hardened whale oil melted together as a vitamin A addition to their fundamental food, the animals died from deficiency of vitamin A. Con-

sequently, the vitamin A of the butter fat must have been destroyed or the whale blubber had a toxic effect on the animals. Later experiments on rats, however, proved (Fridericia, 1924-25) that whale blubber did not act as a toxic agent on the animals, but on the butter fat. The vitamin A deficiency did not occur when butter fat and hardened whale oil were not melted together but given separately, or when the hardened whale oil was kneaded together with the fundamental food and butter fat given separately.

Fridericia examined other hardened fatty substances, coconut oil and hemp-seed oil, in the same manner, but neither of them displayed any destructive power on vitamin A. Later he demonstrated that lard, which had been heated to 102-105°C. during 24 hours on a flat tin tray in the presence of air, when kneaded together with butter fat made this latter ineffective as a vitamin

TABLE IV.

Fatty mixture.	Petroleum ether.	Mixture.	Reaction.
<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	
2.5	2.5	50	0
1.25	3.75	25	0
1.00	4.00	20	0
0.50	4.50	10	0

A-supplementing factor. Lard not having been heated in this way was devoid of this power.

I made a mixture of equal parts of the previously tested butter fat, which yielded a positive sulfuric acid reaction, and lard, which had been heated to 102°C. during 24 hours.

After 10 minutes, the time needed for the lard to act on the butter fat, the experiment was started.

A few seconds after the sulfuric acid had been added, but later than the purple color generally appears, the solution became brown, the intensity varying according to the concentration. At a lower concentration a brown deposit appears after a lapse of about 18 hours, while the upper part of the solution is colorless. I repeated the experiment several times; each time obtaining the same result.

I next tried a mixture of butter fat and lard, produced in the same manner, but not having been heated.

As it may be of interest to know the proportions of the mixture of butter fat and heated lard not yielding a sulfuric acid reaction, the following experiments were performed.

With a mixture of 20 per cent of heated lard and 80 per cent of butter fat, the reaction is weakly positive. With a lesser proportion of lard, it is distinctly positive.

Furthermore, it appeared that, even if the mixture of the two substances is made directly in the test-tube and the experiment

TABLE V.

Petroleum ether.	Mixture.	Reaction.
<i>cc.</i>	<i>cc.</i>	
4	1	+

TABLE VI.

Petroleum ether.	Butter fat.	Lard.	Reaction.
<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	
4.0	0.75	0.25	0
4.0	0.80	0.20	(+)
4.0	0.90	0.10	+

TABLE VII.

Petroleum ether.	Butter fat.	Reaction.
<i>cc.</i>	<i>cc.</i>	
4.0	1.0	0
3.75	1.25	0
4.50	0.50	0

immediately undertaken, the result is the same as that shown in Table IV.

In order to find out whether the reaction would disappear by heating the butter fat, the following experiment was made.

A sample of the previously positively reacting butter fat was heated on a flat tin tray for about 4 hours to about 100°C. in the presence of air.

The same brown color appeared in this experiment as when lard was heated and when butter fat and heated lard were mixed; but never an indication of the purple color.

DISCUSSION.

From my experiments and those of other investigators, it appears that butter fat gives a reaction with sulfuric acid and, compared with liver oil, a stronger one than ordinarily recorded. In this connection it certainly is of consequence that the butter fat has been derived from grass butter (Drummond, Coward, and Watson, 1921), whereas the samples of oil, employed by me, had been left standing for a long time, with the result that the chromogenic substance had decreased.

It also turns out that when butter fat is mixed with lard which had been oxidized by heating to 100–102°C. in the air during 24 hours, its power of yielding a sulfuric acid reaction decreases at the same time as the growth-promoting potency disappears. It is to be supposed that substances arise in the lard capable of destroying vitamin A as well as the chromogenic substance in the butter fat, and that these destructive agents are probably peroxides (Fridericia, 1924–25). It is at any rate most probable that the destruction is effected by oxidation, and as a mixture of butter fat and non-heated lard does not prevent the reaction, the destructive substance must be due to the heating on exposure to the air.

Further, it appears from Table VI that the action is quantitative. If the quantity of heated lard does not surpass 20 per cent of the total quantity of fatty substance, the reaction is yielded, although much less pronounced than in pure butter fat or in a mixture of butter fat and non-heated lard. Whether this is altered when the mixture is left standing in order that the fatty substance may have more time to act upon the chromogenic substance of the butter fat, I have not yet examined. It is, however, hardly probable, as an experiment of mixing butter fat and heated lard in a test-tube and directly performing a test for sulfuric acid proved the destruction is instantaneous.

As may be seen, my experiments, as many other researches, serve to demonstrate that there exists an intimate association between the chromogenic substance and the growth-promoting power. They also show that as the growth-promoting power disappears in a mixture with heated lard, so the ability for a typical reaction with sulfuric acid likewise disappears.

SUMMARY.

1. Butter fat gives a reaction with sulfuric acid but not in such low concentrations as cod liver oil, and color indices do not attain so high a value.
2. The lard examined does not give a reaction with sulfuric acid.
3. A mixture of equal parts of butter fat and non-heated lard gives a reaction with sulfuric acid.
4. When lard is heated to 100–102°C. during 24 hours in the air and then mixed with lard, the mixture does not give a reaction with sulfuric acid. The growth-promoting power disappears simultaneously, as shown by Fridericia (1924–25).
5. If the heated lard amounts to less than 20 per cent of the mixture, the only result is a decrease in the intensity of the color index at the reaction with sulfuric acid.
6. When butter fat is heated 4 hours in the air its ability for a typical sulfuric acid reaction disappears.

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THE METABOLISM OF TRYPTOPHANE.

I. THE SYNTHESIS OF RACEMIC Bz-3-METHYLTRYPTOPHANE.

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INTRODUCTION.

In view of the complete dependence of the animal organism on tryptophane for maintenance, and of the relation which may exist between it and the iodine-containing active principle of the thyroid isolated by Kendall, it is highly desirable that further attempts should be made to elucidate the problem of the normal intermediate metabolism of this amino acid.

Knowledge of the changes involved in the oxidation of tryptophane within the animal body is entirely lacking. In the human organism it appears to be completely burnt, and only those products are found in the urine which are exogenous in character and due to bacterial decomposition in the intestine prior to absorption. The only known product of its endogenous catabolism is kynurenic acid, which has been observed in the case of the dog, rat, and rabbit. This acid is not a product on the main line of the destructive breakdown of tryptophane and it is doubtful if it is produced

in the intermediate metabolism of those animals which do not normally excrete it. Beyond this, nothing definite is known of its formation from tryptophane.

The subject is one of more than purely biochemical interest, for there are indications in the literature that the elucidation of the problem of the intermediate metabolism of this amino acid may be of considerable value to the pathological chemist. Eppinger (1910), for example, found that the urine of a patient with melanosarcoma gave intense reactions for indole and tryptophane, and that tryptophane-feeding resulted in a great increase in melanuria. From the evidence at his disposal he concluded that, in these cases, the pyrrole ring did not undergo the usual normal oxidation, and instead it was reduced, methylated, and finally conjugated with sulfuric acid to form an ethereal sulfate of methylpyrrolidine-hydroxy-carbonic acid. To this compound he assigned the formula $C_6H_{12}N_2SO_4$. Abderhalden (1912) also found in the urine of a melanuric a substance rich in tryptophane, while Fränkel (1912), investigating cancerous tissue, came to the conclusion that cancers were often defective in tryptophane and that, while normal squamous epithelium was rich in tryptophane, a squamous cell carcinoma contained little or none of this amino acid. If, therefore, as appears from these cases, there is some close connection between tryptophane and the body pigments, the elucidation of the mechanism of the normal oxidation of tryptophane may throw valuable light on these and allied pathological conditions.

HISTORICAL.

Historically, the literature on the subject deals mainly with the chemistry of kynurenic acid and with investigations of the mode of its formation from tryptophane. In more recent years the technique of feeding experiments has been considerably improved and several interesting facts have been gained by this method. Workers in this and in other fields are, however, considerably handicapped by the difficulty of synthesising derivatives of tryptophane which may be presumed to be on the main line of breakdown of this amino acid.

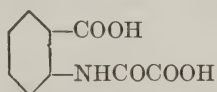
Kynurenic acid was first isolated as a product of animal metabolism by Liebig (1853) from the urine of the dog, and to it he assigned the formula

$C_{16}H_7NO_5$. Hofmeister (1881), after much careful research, failed to find it in human urine, while Capaldi (1897) was unable to detect it in the case of the wolf and of the fox. Attempts were made by Mendel and Jackson (1898) to detect its presence in the urine of the cat under various dietary conditions, but without success. Working under Mendel's direction Swain (1905) discovered it in the urine of the coyote—*Canis ochropus* Eschscholtz—an animal resembling the dog, but with many of the characteristics of the fox and of the wolf.

Working in another direction Schneider analysed the acid and put forward for it the formula $C_{20}H_9NO_6$, while Schmiedeberg and Schultzen (1872), from results obtained by analysing the free acid and also its barium salt, concluded that the formula was $C_{20}H_{14}N_2O_6 \cdot 2H_2O$. They also showed that the acid, heated at $150^\circ C.$, became anhydrous and that, on melting, carbon dioxide was split off, with the formation of a substance which they called kynurin. According to these workers kynurin and its platinum salts on analysis gave results in accordance with the formula $C_{18}H_{14}N_2O_2$ for the former compound.

Kretschy (1881, 1883, 1884) took up the question and, after assigning the formula $C_{18}H_{14}NO_3 \cdot H_2O$ to the acid, made the following observations regarding kynurin and kynurenic acid: (a) kynurin is of a phenolic nature; (b) kynurin on distillation with zinc dust yields quinoline; (c) kynurenic acid on distillation with hydrochloric acid and zinc dust yields quinoline; (d) kynurenic acid on oxidation with alkaline permanganate is converted into oxalic acid and an acid to which he gave the name kynuric acid; (e) kynuric acid on hydrolysis with water gave oxalic acid and *o*-aminobenzoic acid; while with potassium hydroxide and potassium carbonate aniline was formed.

His analysis of kynurenic acid gave results agreeing with the formula $C_9H_7NO_5$. These observations made it clear that kynuric acid has the structure



and that kynurenic acid is hydroxyquinoline carboxylic acid.

Later Wenzel (1894) prepared kynurin from cinchonic acid and showed that it was γ -hydroxyquinoline. Camps (1901) confirmed this work by synthesising kynurin from formyl-*o*-aminoacetophenone. He, moreover, synthesised γ -hydroxy- α -carboxy-quinoline (m. p. = $290^\circ C.$) and γ -hydroxy- β -carboxy-quinoline (m. p. = 266 to $267^\circ C.$) and, from a comparison of these melting points with those given by Schmiedeberg and Schultzen, and by Kretschy for the natural acid, he concluded that Liebig's kynurenic acid was a γ -hydroxy- β -carboxy derivative.

The investigation of the formation of kynurenic acid in the animal body had in the meantime received attention at the hands of Voit and Reidener,

Hauser, Nigeller, Mendel, Giacosa, and Bauman and Solomno, but it was not until 1904, when Ellinger carried out his feeding experiments, that tryptophane was proved to be the precursor of kynurenic acid. Ellinger went a stage further and put forward the view that the single nitrogen atom of kynurenic acid was represented by the amino nitrogen of tryptophane, a view which gained considerable support when it was shown by Homer (1915) that kynurenic acid was 4-hydroxyquinoline-2-carboxylic acid and not 4-hydroxyquinoline-3-carboxylic acid as believed by Camps.

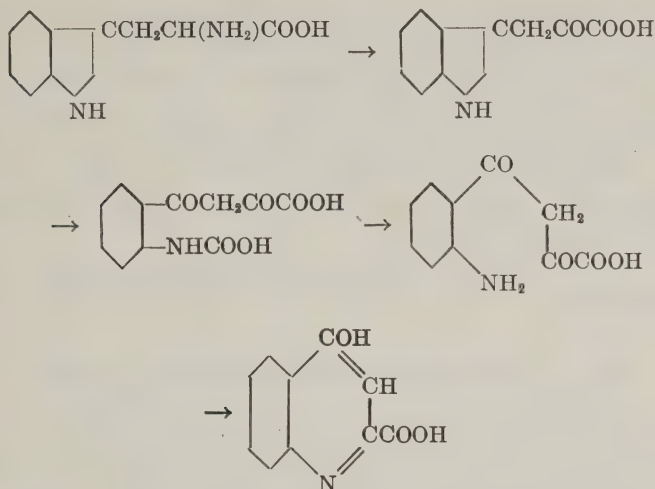
Homer's conclusion as to the chemical constitution of kynurenic acid has been verified by Späth (1921), and by Besthorn (1921). Both of these workers prepared derivatives of the natural and synthetic acids and found them identical. Späth also showed that kynurenic acid was rapidly freed from protein compounds by conversion into its methyl ester, a process which should, though it had not apparently occurred to the worker, considerably simplify the present method, originated by Capaldi (1897), of estimating kynurenic acid.

Returning to the consideration of the question of the mode of formation of kynurenic acid from tryptophane, it will be seen that there is another possibility besides that suggested by Ellinger; namely, the formation of the quinoline ring of the acid by the entrance of an additional carbon atom into the indole ring. To support this view there is a certain amount of justification, chemically; *e.g.*, β -chloroquinoline is obtained together with indole-3-aldehyde by the action of chloroform and potassium hydroxide on indole. There is, however, one serious objection to this hypothesis as Barger and Ewins (1917) pointed out in the instructive foreword to their paper on Pr-2-methyltryptophane. Oxidation of the side chain of the tryptophane molecule should lead to the production of a carboxyl group in position 3 (pyrrole ring), while in the kynurenic acid molecule it is present in position 2 (quinoline ring). This would involve a wandering of the COOH group from position 3 to 2 during the transformation, a movement which is unlikely. This objection in turn has been countered by a mechanism adopted by Ellinger and Matsuoka, to which reference will be made later.

Clearly, if a substituted kynurenic acid could be obtained by feeding a substituted tryptophane, it would be possible to decide between the two hypotheses, and it was on these lines that Ellinger and Matsuoka (1914) and Barger and Ewins (1917) fed Pr-2-methyltryptophane, the former to a rabbit, the latter to a puppy. In neither case was an excretion of a derivative of kynurenic acid observed. Barger and Ewins in the conclusion of their paper were, however, inclined to the view that in natural tryptophane the pyrrole ring was eliminated, while in 2-methyltryptophane this elimination is prevented by the protective action of the methyl group.

More recently, Ellinger and Matsuoka (1920) synthesised indole-3-pyruvic acid, administered it intravenously to rabbits, and observed that, like tryptophane, it was converted into kynurenic acid, though not to an equivalent extent. The authors put forward the tentative suggestion

that this compound forms the first stage in the mechanism of the transformation of tryptophane into kynurenic acid which is as follows:



The authors, however, drew attention to the fact that other ketonic acids, *e.g.* pyruvic acid and phenylpyruvic acid, yield the corresponding amino acids, alanine and phenylalanine, when perfused through the surviving liver, and that indole-3-pyruvic acid may follow a similar course in the body. If this is so, tryptophane would be the first stage of the transformation of indole-3-pyruvic to kynurenic acid; in other words, the original problem comes into being again. In the same paper Ellinger and Matsuoka described the synthesis of quinoline-2-carboxylic acid. This, when fed to rabbits, was recovered from the urine partly unchanged and partly conjugated with glycine. "Hence it is safe to assume that kynurenic acid does not result from a direct hydroxylation of a quinoline derivative by the reaction comparable to the formation of phenolic substances from benzene derivatives."¹

Before concluding this historical sketch a brief reference must be made to the experiments of Abderhalden, London, and Pincussohn (1909), from which they concluded that the liver was not the seat of the formation of kynurenic acid. Much more recently Matsuoka and Takemura (1922) perfused a dog's liver with blood containing either tryptophane or indole-pyruvic acid, and they observed, in both cases, formation of kynurenic acid. As this result directly contradicts the above observation of Abderhalden and his colleagues, it is evident that the work requires repetition.

¹ Dakin (1922), p. 96.

Method.

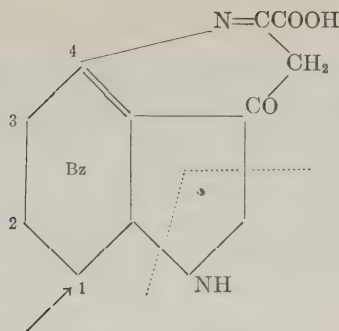
Of the various ways of attacking such a problem as the intermediate metabolism of tryptophane, the two most likely to yield results are: (1) the feeding or injection of a substituted tryptophane to a puppy or rabbit, followed by the examination of the urine for possible derivatives; and (2) the perfusion of tryptophane itself, and its possible degradation products, through the isolated surviving liver, so to overcome the tendency of the intact animal to effect complete oxidation.

The scheme of work in hand includes the application of both these methods.

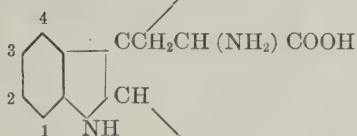
Reasons for the Selection of Bz-3-Methyltryptophane.

For the purpose of the first method a suggestion, originally made by Barger and Ewins (1917), to the effect that a tryptophane substituted in the benzene ring would be the most suitable, was adopted. The synthesis of such a tryptophane is described in this thesis.

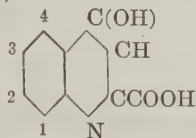
With four unsubstituted hydrogen atoms in the benzene nucleus of tryptophane, four mono- and six di-, substituted tryptophanes, as well as three naphthalene derivatives, are theoretically possible. The parent substances of all these compounds are the correspondingly substituted indoles, and since, of these, the mono- substituted indoles have been more fully investigated, more attention was directed to these, than to the others, as a possible line of attack. It was soon evident, however, from several considerations, that the number of mono- substituted compounds available for the present work was severely limited to one. Leaving out of consideration for the moment the possibilities of the scheme of degradation (see page 499) adopted by Ellinger and Matsuoka, and referring to the following diagram, which shows the two older alternative theories of the transformation of tryptophane to kynurenic acid, it will be seen that:



By pyrrole ring cleavage and union of amino N to the benzene ring (Theory I).



By enlargement of indole ring to quinoline ring (Theory II).



(a). A substituent in 4 is ruled out as it would prevent ring formation if the transformation occurs according to Theory I.

(b). A substituent in 2 would form the same substituted kynurenic acid whether the transformation occurs according to either theory, and therefore cannot be used.

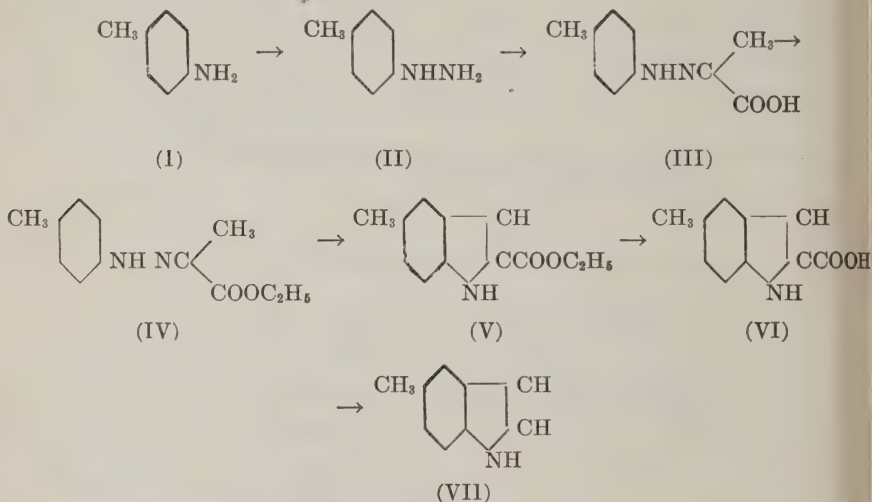
(c). The number of remaining substituted tryptophanes possible is further limited by the fact that 7-methylindole, the parent substance of Bz-1-methyltryptophane, still awaits synthesis. Raschen (1887), who synthesised 5-methylindole, was unable to isolate 7-methylindole from the mass obtained on melting 2-carboxy-7-methylindole and Harington, working under Barger, confirmed this difficulty.² It is possible that this step may be successfully performed by the method of Kermack, Perkin, and Robinson to which reference will be made later, and experiments are being carried out with this in view.

The theoretically possible methylindoles are thus cut down to one, *viz.* 5-methylindole, and from this the synthesis of Bz-3-methyltryptophane has been accomplished.

² Personal communication.

Synthesis of Bz-3-Methyltryptophane.

The main difficulties in this connection, which at first sight appear formidable, are firstly, the smallness of the yield of 5-methylindole, synthesised by Raschen (1887) according to the following method (Fischer's synthesis),

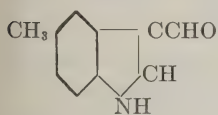


and, secondly, the poor yield of the corresponding aldehyde. The starting material must, therefore, be cheap. Partly on this account, and partly because *p*-toluidine gives the hydrazine in good yield, which is not the case with the xylidines and dimethoxyanilines, it was decided to repeat Raschen's work and endeavour to improve his yields of 5-methylindole.

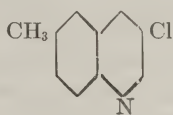
The first point investigated was the formation of 2-carbethoxy-5-methylindole (V). Here it was found unnecessary to isolate the ester of the pyruvic acid-*p*-tolylhydrazone (IV) and then heat this with zinc chloride to obtain 5-methylindole-2-carboxylic ester (V). By dissolving the hydrazone in absolute alcohol and bubbling dry hydrogen chloride rapidly through the solution, esterification and indole ring formation were found to take place with the separation of ammonium chloride. On pouring the dark brown solution so obtained into excess of water, the ester separated out in good yield as a semicrystalline mass, which was most easily purified by distillation in a high vacuum.

The next difficulty lay in the small yield of 5-methylindole (VII) obtained from 5-methylindole-2-carboxylic acid (VI). In the original method, this was done by heating the dry acid in a boiling tube at the temperature at which it melted. The yield obtained in trial experiments was never as high as Raschen claimed; *viz.*, 20 to 30 per cent. Attempts to improve the yield by heating the acid in dry glycerol at 220°C. were not successful. Finally, the method adopted by Kermack, Perkin, and Robinson (1922), of heating the dry ammonium salts, was tried with the result that a yield of 5-methylindole, equivalent to 57 per cent of the theory, was obtained. The procedure has also this advantage, that the ether, from which the ammonium salt is precipitated, retains the impurities in solution.

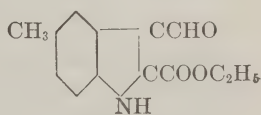
In the synthesis of 5-methylindole-3-aldehyde (VIII) Ellinger and Flamand's (1907) modification of the Tiemann-Reimer reaction gave the aldehyde in only an 11 per cent yield which, however, becomes 23 per cent if the 4.6 gm. of methylindole recovered is taken into account. Gattermann's hydrocyanic acid method, used with such success by Barger and Ewins (1917) in making 2-methylindole-3-aldehyde, is not applicable here. It was hoped that a better result would be obtained by means of Majima and Kotake's (1923) modification of the Grignard reaction, using anisole instead of ether as a solvent. Here the primary yield of aldehyde was greater (approximately 19 per cent), but no unchanged indole could be recovered. A third method of attack was attempted, and was as follows:



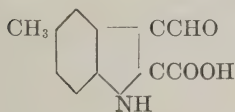
(VIII)



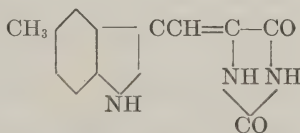
(IX)



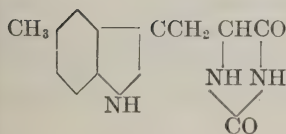
(X)



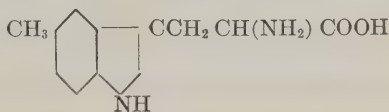
(XI)



(XII)



(XIII)



(XIV)

The ethyl ester of 5-methylindole-2-carboxylic acid (V) was converted into the corresponding aldehyde (X) followed by hydrolysis to the acid (XI). Heating the dry ammonium salt of this acid in a high vacuum, however, resulted in the formation of a deep red tar and only a slight trace of the required 5-methylindole-3-aldehyde could be isolated. Of the two first methods Ellinger and Flamand's was finally adopted.

Attempts were now made, according to the method of Ellinger and Flamand, to condense the aldehyde so obtained with hippuric acid (Perkin's synthesis). The operation, carried out under varying conditions, always resulted in the formation of so much tar that it was impossible to obtain the az-lactone in a degree of purity sufficient for purposes of analysis. Moreover there arises the question of the insolubility, always an important point in synthetic organic chemistry, of the az-lactone. As a consequence it was decided to attempt the method used by Majima and Kotake (1922), who condensed their indole-3-aldehyde with hydantoin and obtained a compound soluble in 0.5 N sodium hydroxide. Success attended attempts in this direction.

The resulting (5-methyl)-indolalhydantoin (XII) dissolved easily in dilute sodium hydroxide, and in solution was reduced with 2.5 per cent sodium amalgam, yielding 5-methylindolylhydantyl methane (XIII). Hydrolysis of this compound by baryta completed the synthesis.

EXPERIMENTAL.

p-Tolylhydrazine (Formula II).

This compound was prepared by Raschen who gave no details in his paper. The following procedure was found to be suitable.

Vigorous stirring and a temperature of 0–2°C. at all stages of the preparation were found to be essential for good yields. *p*-Toluidine (53.5 gm.) was slowly added to hydrochloric acid (500 cc. of $d = 1.16$), and at the above temperature a semicrystalline mass (*p*-toluidine hydrochloride) separated out. Sodium nitrite (34.5 gm. in 150 cc. of water) was then slowly added. Stannous chloride (290 gm.), dissolved in hydrochloric acid (250 cc.) and well cooled, was next run in when a heavy white precipitate separated out. The reaction mixture was allowed to stand in a cold place overnight; the white precipitate was then collected, well pressed,

and dried on a porous plate. When dry, it was shaken with sodium hydroxide (400 cc. of 25 per cent) and ether (400 cc.), and the ethereal layer separated. The ethereal extraction was repeated three times, and the combined extracts were dried over anhydrous sodium sulfate overnight. On distilling off the ether on the water bath, an oil remained which solidified to a mass of long, practically colorless, needles, weighing 52.7 gm. It melted at 60°C. (melting point of *p*-tolylhydrazine = 61°C.) and was sufficiently pure for the purpose of the next experiment.

Pyruvic Acid-p-Tolylhydrazone (Formula III).

p-Tolylhydrazine (52 gm.) was dissolved in 1,500 cc. of approximately $N/3$ hydrochloric acid, and 30 cc. of pyruvic acid ($d = 1.26$) dissolved in 250 cc. of water were then slowly added under vigorous stirring. The yellow crystalline condensation product immediately separated, and towards the end of the experiment the mixture became so thick that complete mechanical stirring was impossible. It was then filtered on the Büchner funnel, the solid air-dried, and crystallised from hot 80 per cent alcohol. The yellow needles so obtained melted at 159°C. (Raschen gives 158–160°C., uncorrected) and weighed 72 gm. (88 per cent of theory).

2-Carbethoxy-5-Methylindole (Formula V).

This compound was not obtained directly by Raschen from pyruvic acid-*p*-tolylhydrazone, but from its ethyl ester by fusion with zinc chloride. On repeating the experiment the yield of the indole compound was found to be poor, and, moreover, it had the disadvantage that only some 5 gm. could be worked up at a time. The following method was then tried and gave very satisfactory results.

Pyruvic acid-*p*-tolylhydrazone (30 gm.) was dissolved in absolute alcohol (250 cc.), the temperature of the solution being maintained at 65–70°C. Meanwhile, a rapid stream of dry hydrogen chloride was bubbled through the solution. After 45 minutes a white crystalline precipitate, consisting of ammonium chloride, began to separate. The supply of hydrogen chloride was stopped at the end of 2 hours, the solution allowed to cool, and then poured into a large volume of water. The semicrystalline brown mass was separated on a Büchner funnel, dried overnight in the air, and dis-

tilled in a vacuum. At 4 mm. the ester distilled at a temperature of 236°. The distillate rapidly condensed to a mass of colourless plates, which melted at 163°C. (Raschen gives 158–160°C). Yield 19 gm. (60 per cent of theory).

5-Methylindole-2-Carboxylic Acid (Formula VI).

This acid was obtained according to Raschen's method by hydrolysing the ethyl ester just described with a 6 per cent alcoholic potassium hydroxide solution for 30 minutes. Water was then added, most of the alcohol sucked off, and the solution made acid, when the 5-methylindole-2-carboxylic acid separated as a sandy powder. It was not removed from the solution from which it was precipitated, but the whole mixture was extracted with ether as in the next experiment.

5-Methylindole (Formula VII).

For reasons indicated in the introduction, Raschen's method of obtaining this substance from 5-methylindole-2-carboxylic acid was replaced by one similar to that used by Kermack, Perkin, and Robinson, and was as follows:

The mixture obtained on acidifying the product of hydrolysis of the 2-carbethoxy-5-methylindole was repeatedly extracted with ether until a sample of the extract gave no further residue on evaporation. The combined ethereal extracts were dried overnight over anhydrous sodium sulfate. The ethereal solution was then filtered off and a rapid stream of dry ammonia was passed through it. The ammonium salt of the 5-methylindole-2-carboxylic acid immediately began to separate as a powder, slightly yellow in colour. After saturation the mixture was allowed to stand 2 to 3 hours and the ether then sucked off. The dry ammonium salt (10 gm.) was placed in a round-bottomed flask (1 liter), the neck of which was closed with a long spiral air-cooled condenser, and heated on an oil bath at 230–240° for 30 minutes. The condenser was disconnected, some water added to the contents of the flask which were then steam-distilled till the distillate gave only a faint colouration with Ehrlich's reagent. The distillate on cooling deposited the 5-methylindole in long colourless needles, which melted at the correct temperature (58.5°C.). Yield 3.9 gm. (57 per cent of theory).

*5-Methylindole-3-Aldehyde (Formula VIII).**(a). Ellinger and Flamand's Method.*

5-methylindole (9 gm.) was dissolved in a mixture of 96 per cent alcohol (100 cc.), chloroform (36 cc.), and water (20 cc.) in a 500 cc. conical flask, which was provided with a reflux condenser, a powerful mechanical stirrer, and a dropping funnel. The temperature of the solution was raised until it was just boiling, and a solution of potassium hydroxide (25 gm.) in water (25 cc.), made up to 250 cc. by the addition of alcohol (96 per cent), was gradually and regularly dropped in over a period of $2\frac{1}{2}$ hours. The whole mixture was then vigorously refluxed for 30 minutes. The alcoholic solution was poured off from the potassium chloride which had separated, the potassium chloride several times washed with the ether, and the washings added to the original alcoholic solution. The alcohol ether was distilled from the solution contained in a round-bottomed flask (1 liter) and the residual oil steam-distilled until the distillate gave only a faint pink colour with Ehrlich's reagent. The liquid remaining in the flask was immediately filtered. The tar which remained was again boiled with water and refiltered; the filtrates were combined and cooled, when the required 5-methylindole-3-aldehyde separated in sheaves, which were only slightly coloured. It was most easily purified by dissolving in boiling aqueous methyl alcohol (50 per cent) and cooling rapidly under the tap with stirring. Yield 1.2 gm.

Nitrogen by micro Kjeldahl.

25.8 mg.: 2.28 mg. N_2 .

$C_{10}H_9ON$. Calculated. N 8.8.

Found. " 8.86.

It melted sharply at $148^\circ C$. and was very soluble in hot petroleum ether, b.p. $110-120^\circ C$.

3-Chloro-6-Methylquinoline (Formula IX).

This compound passed over together with the unchanged 5-methylindole when the product of the action of chloroform and potassium hydroxide on the latter was distilled in steam as described above. The distillate, about 4 liters in volume, was extracted several times with ether, the combined ethereal extracts thoroughly shaken with several small quantities of dilute hydro-

chloric acid (10 per cent), dried, and evaporated, when almost pure 5-methylindole (4.6 gm.) remained. The acid extract of the ethereal solution was made alkaline by the addition of sodium hydroxide and allowed to cool, when 3-chloro-6-methylquinoline separated out. It was most easily purified by dissolving the impure quinoline compound in dilute hydrochloric acid, filtering on a Büchner funnel, and neutralising, when almost colourless needles were obtained. The supernatant liquid was sucked off, and the solid recrystallised from methyl alcohol (40 per cent). (Found Cl 19.9. $C_{10}H_8NCl$ requires Cl = 20 per cent.) 3-chloro-6-methylquinoline melted at $85.5^{\circ}C.$, and had a pleasant smell, resembling lilac.

(b). *Majima and Kotake's Method.*

Well dried magnesium powder (2.4 gm.) was covered with freshly distilled anisole (10 cc.) in a conical flask (150 cc.), fitted with a reflux condenser and closed to the air by a CO_2 absorption tube; ethyl iodide (16 gm.) added, and the magnesium activated by the addition of a very small crystal of iodine. When most of the magnesium had dissolved, the flask and its contents were cooled in an ice-sodium chloride bath, and under vigorous shaking 5-methylindole (6.5 gm.) was added in small portions. The total volume of gas, consisting of ethane, given off during the reaction measured 0.98 liters. To the thick colourless syrup now remaining in the reaction flask, again well cooled, excess of ethyl formate (20 cc.) was slowly added under constant shaking. The colour of the liquid immediately changed to a brilliant red, which, however, faded, though not completely, with further additions of ethyl formate. The reaction mixture then stood for 30 minutes; ice-cold water was now dropped in under shaking, and a slight excess of dilute acetic acid. Contrary to the findings of Majima and Kotake in their work on indole-3-aldehyde, no precipitate separated at this stage. The liquid so obtained was shaken several times with ether, the combined ethereal extracts were washed with sodium bicarbonate solution, the ether was evaporated, and the residue steam-distilled until the distillate gave no colouration with the Ehrlich reagent. The aqueous solution was filtered hot, and the red tar remaining was once more boiled with water and refiltered. The combined filtrates on cooling deposited 5-methylindole-3-aldehyde. Yield 1.15 gm.

The crystals were not coloured as in the previous experiment; m.p. 151°C.

Nitrogen by micro Kjeldahl.

26.5 mg.: 2.3 mg. N₂.

C ₁₀ H ₉ ON.	Calculated.	N 8.8.
	Found.	" 8.69.

2-Carbethoxy-5-Methylindole-3-Aldehyde (Formula X).

This substance was prepared according to a modification of Gattermann's method, described by Adams and Levine (1923), from 2-carbethoxy-5-methylindole.

2-carbethoxy-5-methylindole (10 gm.) was dissolved in dry chloroform (200 cc.) in which was suspended dry zinc cyanide (8.2 gm.). The conical flask containing this mixture was fitted with a cork carrying a lead-in from a hydrogen chloride generator, and also a reflux condenser which was closed to the air by a chain of wash bottles containing concentrated sulfuric acid and sodium hydroxide solution.

The contents of the flask were cooled to 0°C. by immersion in an ice-salt bath and dry hydrogen chloride was bubbled through. After 20 minutes a dark oil separated which gradually solidified to a yellow substance. After 1½ hours the temperature was raised to 30°C. and the hydrogen chloride passed in for another 30 minutes. The flask was then allowed to stand overnight, when the supernatant liquid was poured off, the crystals were washed several times with chloroform, water (100 cc.) was added, and the mixture heated on the water bath for 30 minutes. The imino compound quickly decomposed as evidenced by the disappearance of the yellow colour. On cooling, the liquid was sucked off and the colourless solid weighing 8 gm. was crystallised from alcohol (70 per cent) from which it separated on rapid cooling in fine needles. (Found N = 6.15. C₁₃H₁₃O₃N requires N = 6.06).

2-Carbethoxy-5-methylindole-3-aldehyde melted at 189°C. and is readily soluble in absolute alcohol and in ether. From hot xylene it crystallised in small plates.

2-Carboxy-5-Methylindole-3-Aldehyde (Formula XI).

In the preparation of this substance 2-carbethoxy-5-methylindole-3-aldehyde was hydrolysed by a concentrated aqueous

solution of sodium hydroxide (40 per cent) for 15 minutes and the resulting solution acidified. The supernatant fluid was sucked off on a Büchnerfunnel; the solid was dissolved in dilute ammonium hydroxide, boiled with animal charcoal, and refiltered. On the neutralisation with hydrochloric acid, the 2-carboxy-5-methylindole-3-aldehyde separated as a sandy, slightly yellow powder. It was soluble in the usual organic solvents. From methyl alcohol, on quickly cooling, it separated in clusters of colourless needles; from xylene it crystallised in small cube-like crystals.

Nitrogen by micro Kjeldahl.

22.3 mg.: 1.52 mg. N₂.

C₁₁H₉O₃N. Calculated. N 6.9.

Found. " 6.81.

On heating it turned brown at 235°C. and melted at 254–255°C. with violent decomposition.

2 gm. of 2-carboxy-5-methylindole-3-aldehyde were heated in a vacuum distillation flask (pressure = 1.2 mm. Hg). At 220°C. the compound began to sublime. The small amount of substance which collected in the receiver was dissolved in ether and the ether evaporated. The colourless crystalline product so obtained melted at 148°C., indicating that it was probably 5-methylindole-3-aldehyde. Sufficient of the substance was unobtainable for the N₂ estimation to verify this conclusion.

The residue in the distillation flask consisted of a dark red tar which, though very soluble in acetone and ether, could not be crystallised for examination.

(5-Methyl)-Indolalhydantoin (Formula XII).

5-Methylindole-3-aldehyde (6.3 gm.), hydantoin (4.1 gm.), and freshly fused sodium acetate (3.75 gm.) were thoroughly ground up together, and added to freshly distilled acetic anhydride (14.5 cc.) in a large boiling tube, fitted with a reflux condenser. The temperature of the mixture was maintained at 105–107° on an oil bath for 30 minutes. Within 5 minutes, the solution turned dark brown, and yellow crystals began to separate. The mass solidified on cooling and was rubbed up several times with water, filtered, and dried. The dry mass weighed 9.5 gm.

When dry, the brownish yellow mass was extracted several times with hot petroleum ether (b.p. = 110–120°) till no further solid separated when the solvent cooled. The partially purified methylindolalhydantoin was shaken with *N* sodium hydroxide when all but a dark brown tar dissolved. The tar was separated by filtration. On acidifying the filtrate with dilute acetic acid, the hydantoin was precipitated as a bright yellow amorphous mass which quickly settled. The solid was sucked dry and crystallised from glacial acetic acid.

Nitrogen by micro Kjeldahl.

0.011 gm.: 1.87 mg. N_2 .

$C_{13}H_{11}O_2N_3$. Calculated. N 17.4.

Found. " 17.0.

The substance melted at 295–298°, and was very insoluble in ether, butyl alcohol, ethyl acetate, and xylene. It crystallised from glacial acetic acid in small cubes.

5-Methylindolylhydantyl Methane (Formula XIII).

3.5 gm. of (5-methyl)-indolalhydantoin were dissolved in 175 cc. of 0.5 *N* NaOH and the solution was stirred mechanically while approximately 140 gm. of 2.5 per cent sodium amalgam, in three portions, were dropped in. After 1½ hours the action ceased, by which time the original brownish yellow colouration had practically disappeared. On careful neutralisation with dilute hydrochloric acid the 5-methylindolylhydantyl methane was precipitated. The crude dry produce weighed 2 gm. and was recrystallised twice from hot water when a mass of colourless needles was obtained. It melted at 206–207°.

Nitrogen by micro Kjeldahl.

9.6 mg. substance: 1.62 mg. N.

$C_{13}H_{13}O_2N_3$. Calculated. N 17.3.

Found. " 16.9.

The substance was insoluble in cold water; soluble in hot water; very soluble in ether and alcohol; and partly soluble in hot benzene.

Bz-3-Methyltryptophane (Formula XIV).

5-methylindolylhydantyl methane (1.2 gm.) was refluxed with 60 cc. of a 60 per cent aqueous solution of barium hydroxide on a sand bath. At the end of an hour evolution of ammonia commenced and continued throughout the experiment. After $6\frac{1}{2}$ hours the mixture was cooled, its volume increased to 350 cc. by addition of water, and carbon dioxide led in to precipitate the barium. The liquid was then heated just to the boiling point and filtered, and the residue repeatedly extracted with hot water until the addition of bromine water gave no further purple colouration to the extract. The combined extracts were made up to 7 per cent with sulfuric acid, and 100 cc. of Hopkin's reagent added. A brownish yellow precipitate immediately commenced to separate. Next morning the mercury sulfate precipitate was sucked dry, washed with distilled water, suspended in 250 cc. of distilled water, and when the supernatant liquid had been made slightly alkaline by addition of barium hydroxide, hydrogen sulfide was passed in under pressure for 3 hours. The mixture was then warmed and filtered, and the mercury sulfide precipitate reextracted with warm water. The combined filtrates, measuring 550 cc., were carefully neutralised with sulfuric acid, refiltered, and then concentrated *in vacuo* at 26°C . until only some 20 cc. of solution remained. Addition of absolute alcohol in excess rapidly precipitated a semi-crystalline mass. This was sucked off and dried. It weighed 0.55 gm. It was redissolved in water, filtered, and precipitated with absolute alcohol when the product was obtained as long glancing platelets which, on recrystallisation from 50 per cent alcohol, melted at $259\text{--}263^{\circ}$. Yield 0.39 gm.

Nitrogen by micro Kjeldahl.

54 mg. substance: 7.12 mg. N.

$\text{C}_{12}\text{H}_{14}\text{O}_2\text{N}_2$.	Calculated.	N 12.84.
	Found.	" 13.2.

Amino nitrogen by Van Slyke.

5.4 mg. substance: 0.335 mg. amino N_2 .

	Calculated.	Amino N 6.42.
	Found.	" " 6.2.

SUMMARY.

Bz-3-methyltryptophane is readily soluble in water and is precipitated from its concentrated solution therein by the addition of absolute alcohol.

Its solution in water gives: (a) a beautiful purple colouration with Hopkins and Cole's reagent, which persists a considerable time if the solution is dilute and is kept cool; (b) a purple colouration with bromine water which is extractable with butyl alcohol; (c) on careful neutralisation, a strong reaction with triketohydrindene hydrate; and (d) is very bitter in taste. This is probably due to its being racemic.

The work recorded above forms the first part of a very much wider investigation. I desire, however, to take this early opportunity of recording my indebtedness to Professor Meakins, who in the first place suggested a piece of research that ultimately led to the present work, and who, also, has placed every facility at my disposal to enable me to carry it out.

I also thank Professor Barger for his readiness on all occasions to discuss my difficulties; his advice and criticism have been invaluable. He, moreover, kindly gave me a supply of *p*-toluidine.

To the Medical Research Council I am indebted for a grant towards maintenance, while a grant from the Moray Fund Grants Committee of the Edinburgh University enabled me to purchase many of the costly materials used in this work.

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METABOLISM STUDIES WITH CHAULMOOGRA OIL.

I. THE INFLUENCE OF CHAULMOOGRA OIL ON CALCIUM METABOLISM.*

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(Received for publication, August 30, 1924.)

Considering the benefit obtained in the treatment of leprosy by Rogers (1, 2), Muir (3, 4), and others by the use of both cod liver oil and chaulmoogra oil, it was thought probable that the latter oil might have a similar action to cod liver oil upon general metabolism. That the calcium metabolism is seriously involved in advanced leprosy is apparent in the dissolution of the bone tissue and subsequent loss of the phalanges. It is logical to conclude that morrhuates are beneficial in advanced leprosy on account of their effect in producing calcium retention. Little is known of the calcium metabolism of early leprosy, but in taking up this problem it seemed likely that chaulmoogra oil, with its ancient empirical records of beneficial effects in the treatment of leprosy, would show a similar effect to cod liver oil in producing calcium retention.

It has been shown that the most active principle of chaulmoogra oil is hydnocarpic acid (5, 6). By repeated fractional distillation of the ethyl esters a fairly pure preparation of ethyl hydnocarpate was made. In the animal experiments reported this was administered in a number of different ways. Use was also made of the crude oil which represents the glyceryl ester of hydnocarpic acid. The other constituents of the oil should not be disregarded, but inasmuch as the results obtained with the oil are similar to those obtained with ethyl hydnocarpate, there appears justification for further emphasis being laid on the hydnocarpate fraction of the oil.

*The data are taken from the thesis of Bernard E. Read presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1924.

EXPERIMENTAL.

A. Effect upon the Urinary Excretion of Calcium in the Rabbit.

Six healthy male rabbits were placed upon a mixed diet of oats and carrots. Two animals were given a diet consisting of 50 gm. of carrots and 75 gm. of oats, which was considered adequate. The others with more capricious appetites were given 50 gm. of carrots and a free supply of oats, but in all instances careful record was kept of the amounts of food consumed.

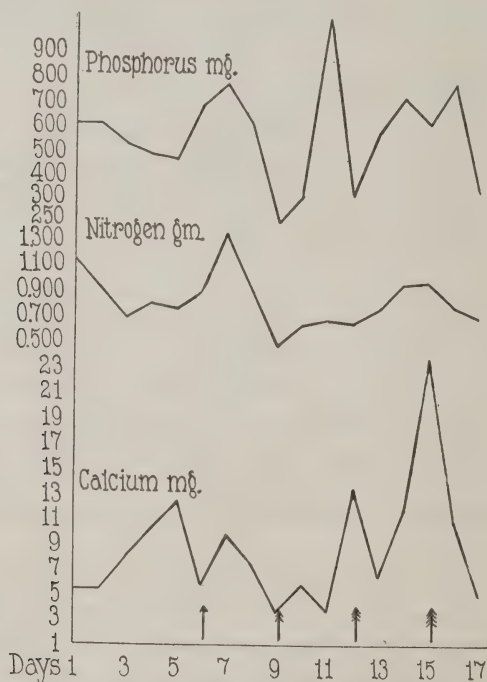


CHART 1. Calcium, nitrogen, and phosphorus excretion after oral administration of hydnocarpus oil. Rabbit 14.

→ 1 cc. →→ 2 cc. →→→ 5 cc. →→→→ 10 cc.

The individual urines were collected daily in 24 hour periods by pressure on the bladder through the abdominal wall. Toluene was used as a preservative. Analyses for calcium were made by McCrudden's method (7). When the volumes were particularly small, precipitation was made in large centrifuge tubes with

subsequent separation and washing, following the usual procedure. The results are given in milligrams of calcium oxide.

The crude oil was introduced orally, subcutaneously, and intraperitoneally. When given by mouth a 10 per cent emulsion of the oil with acacia gum and water was administered by stomach

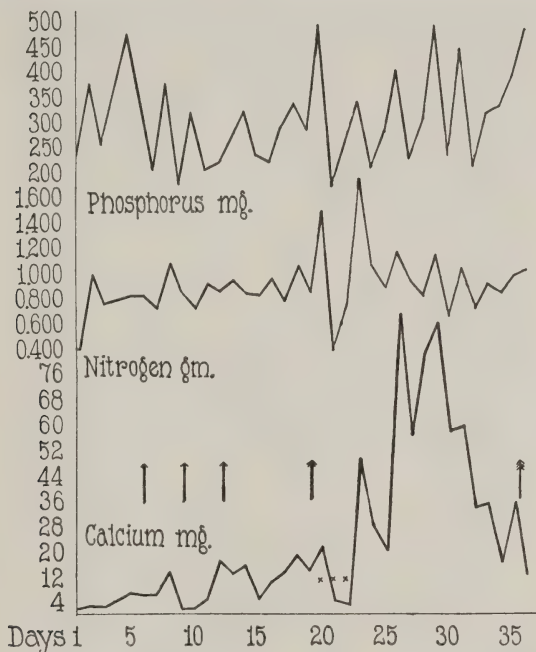


CHART 2. The excretion of nitrogen, calcium, and phosphorus after subcutaneous injections of hydnocarpus oil. Rabbit 11.

→ 1 cc. subcutaneously. →→ 5 cc. intraperitoneally. →→→ 10 cc. orally.
 X practically no food eaten.

tube. This rendered the semisolid oil more convenient for administration, also it presented the oil in a more easily assimilated form.

Such painful local reactions result from subcutaneous injection of the oil, that care was taken in giving ethyl hydnocarpate to mix it with an equal volume of olive oil, and to make special note of any reactions obtained. The abscesses associated with subcu-

taneous administration of the oil were not seen after giving the ethyl hydnocarpate mixture.

Intravenous injection of ethyl hydnocarpate was made very slowly and carefully into the ear vein. Precautions were taken not to produce lung emboli, and also to avoid flooding the ear

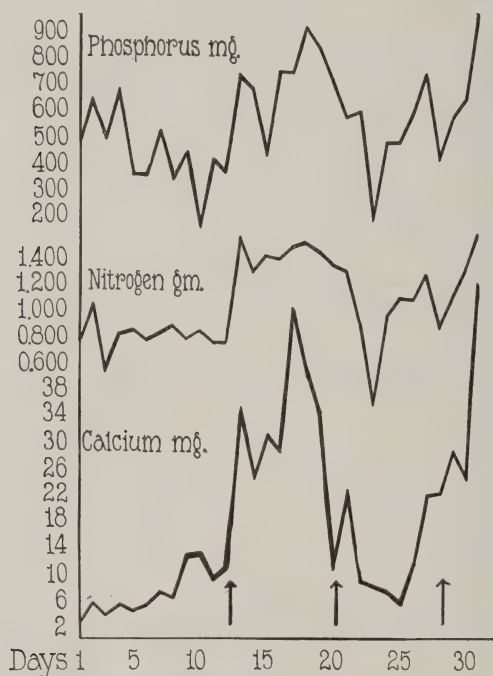


CHART 3. Calcium, nitrogen, and phosphorus excretion after subcutaneous injections of ethyl hydnocarpate. Rabbit 13.

tissues with such an irritating substance. A marked edema was seen in some cases after diffusion of the injection. The exact nature of the acute local reactions produced was not clear, because vascular changes were seen in the ear vessels even when the drug was given orally.

The results show a very marked effect upon calcium metabolism, the most dramatic effect being seen after the intraperitoneal injection of 5 cc. of oil, when the urinary calcium after 7 days rose from 6.9 to 95.2 mg. As presented in Table I the results bring out

clearly the increases produced in the urinary calcium in all of the experiments. Charts 1 to 4 show the detailed daily excretions as compared with the amounts of nitrogen and phosphorus, which will be discussed separately.

A control experiment showed that after an intraperitoneal injection of 5 cc. of olive oil no such phenomenal effect was obtained; on the contrary, there was an insignificant decrease (Table I).

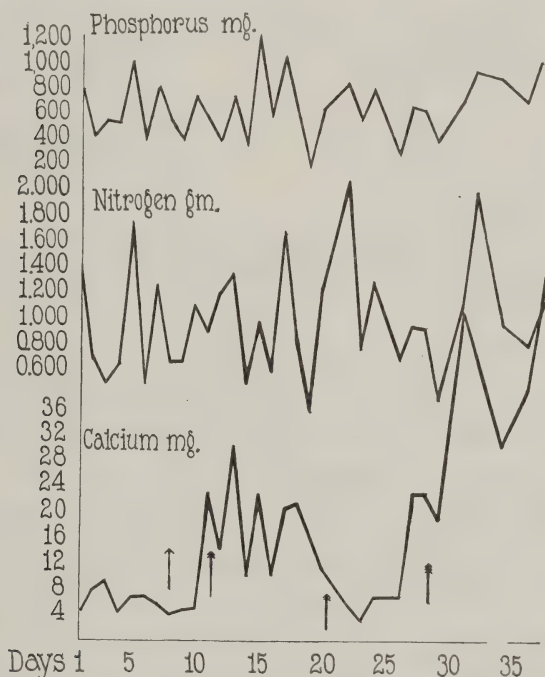


CHART 4. Urinary excretion of calcium, nitrogen, and phosphorus after intravenous injections of ethyl hydncarpate.

→ 0.25 cc. →→ 0.5 cc. →→→ 1.0 cc.

The larger doses of the drug produced marked toxic symptoms, reported upon with many other observations in another paper. Local irritation, anorexia, fever, swelling of the testes, albuminuria, and extreme respiratory distress were sometimes seen after the larger toxic doses, which were fatal in their effect. However, the smaller doses did not show these same untoward symptoms. Other animals similarly treated with small doses survived in *good*

TABLE I.

Urinary Excretion of Calcium by Rabbits after Administration of Chaulmoogra Oil, Ethyl Hydnocarpate, and the Mixed Ester (b. p. 190°C.)

Experiment No.	Method of administration.	No. of days.	Highest out-put. mg.	Average daily out-put. mg.	Increase. mg.
1	By mouth. Fore period. 1 cc. oil on 6th day. 2 " " " 9th " } 5 " " " 12th " } 10 " " " 15th " }	6 6 5	11.3 12.2 22.65	6.95 6.25 10.50	 3.55 -0.7
2	Subcutaneously. Fore period. 1 cc. oil on 6th, 9th, and 12th days. After period.	6 6 6	6.9 16.4 18.5	4.2 7.2 12.3	 3.0 8.1
3	Intraperitoneally. Fore period. 5 cc. oil. After period.	6 6 6	6.9 95.2 59.4	4.2 62.1 37.1	 57.9 32.9
4	Subcutaneously with olive oil. Fore period. Ethyl hydnocarpate, 0.5 cc. " " " 0.5 " with 0.5 gm. CaCl ₂ by mouth.	12 7 10	12.9 50.4 28.6	7.1 40.2 14.8	 33.1 7.7
5	Intravenously. Fore period. Ethyl hydnocarpate, 0.25 cc. on 8th day. Ethyl hydnocarpate, 0.5 cc. on 11th day. Ethyl hydnocarpate 0.5 cc. on 20th day with 0.5 gm. CaCl ₂ by mouth.	8 11 6	8.71 29.75 22.20	5.7 15.5 10.6	 9.8 4.9
6	Intramuscularly with olive oil. Fore period. Mixed ester 0.5 cc. on 5th day. " " 0.5 " " 8th " } " " 0.5 " subcutaneously.	5 5 5	6.85 7.79 7.48	3.8 5.3 4.0	 1.5 0.2

TABLE I—*Concluded.*

Experiment No.	Method of administration.	No. of days.	Highest output.	Average daily output.	Increase.
			mg.	mg.	mg.
	Ethyl hydnocarpate, 1 cc. subcutaneously.	4	6.08	4.0	0.2
	Ethyl hydnocarpate, 1 cc. intravenously.	3	37.20	27.2	23.4
	After period.	4	4.65	2.8	-1.0
7	Control with plain olive oil.				
	Fore period.	5	3.65	2.26	
	5 cc. intraperitoneally.	4	3.35	1.74	-0.52

condition if they were not given lethal doses by mouth or intravenously. When the appetite was affected, after 24 to 48 hours the animal slowly returned to normal, showing a great liking for carrots and totally disregarding the oats provided. It was not until the 6th day after intraperitoneal injections of the oil that the animal ate its usual allowance of oats. It should be noted that, in spite of this, there was a great increase in the calcium output on the days when there was no intake of food.

Routine examination of the urine was made for its action toward polarized light. No rotation was observed, except in one case in which the urine was contaminated with food spilled in the cage. This fact indicates that the hydnocarpates are not eliminated in their original form by way of the kidney. However, dark colored urines were obtained after giving the drug. It will be of interest to see whether these cyclic fatty acids are excreted as ethereal sulfates like the phenols or oxidized in the body like ordinary fats.

The giving of 0.5 gm. of calcium chloride by mouth in Experiments 4 and 5 did not further increase the output of calcium; on the contrary, in both cases there was obtained a decided decrease (Chart 3, 20th day, and Chart 4, 20th day). The entire metabolism appeared to suffer depression as judged by the amounts of phosphorus and nitrogen excreted.

B. Experiments upon Calcium Balance in Dogs after the Administration of the Hydnocarpates.

Methods.—The general plan consisted of metabolism experiments in which the intake and output of calcium, phosphorus, and fat were estimated, first under normal conditions, then with the administration in various ways of chaulmoogra oil and ethyl hydnocarpate.

TABLE II.
Composition of the Daily Diets of Dogs.

	Amount.	N	Fat.	Ca	P	Approximate calories.
Diet A.						
	gm.	gm.	gm.	mg.	gm.	
Meat*.....	250	7.90	9.8	74	1.22	260
Cracker meal.....	200	2.94	1.2	91	1.04	780
Lard.....	20		20.0			186
Agar.....	10			84		
Total.....	480	10.84	31.0	249	2.26	1,226
Diet B.						
Meat*.....	150	5.04	5.85	44.3	0.73	162
Cracker meal.....	150	2.21	0.92	68.7	0.78	585
Butter.....	10±		10.00			93
Agar.....	10			84.0		
Total.....	320	7.25	16.77	197.0	1.51	840
Diet C.						
Meat*.....	100	3.36	3.90	29.5	0.49	108
Cracker meal.....	100	1.47	0.61	45.5	0.52	390
Butter.....	10		10.00			93
Agar.....	5			42.0		
Total.....	215	4.83	14.51	117.0	1.01	591

* Variations in the percentage composition of the different samples of meat purchased have been reckoned in estimating the balances in Tables III and IV.

The calcium in the urine, food, and feces was estimated by McCrudden's methods (7). When the amount of calcium was very small, precipitation was made in centrifuge tubes. The

precipitated solution was centrifuged, and the precipitate washed and titrated in the usual manner.

Phosphorus in the food and feces was determined gravimetrically. The prepared solution of material was precipitated by molybdate, and the solution of the molybdate precipitate in ammonia was treated with magnesia mixture. The resulting precipitate was filtered off, dried, ashed, weighed, and calculated for phosphoric acid (P_2O_5). The urines were titrated with standard uranium solution. The figures for fat represent the total ether

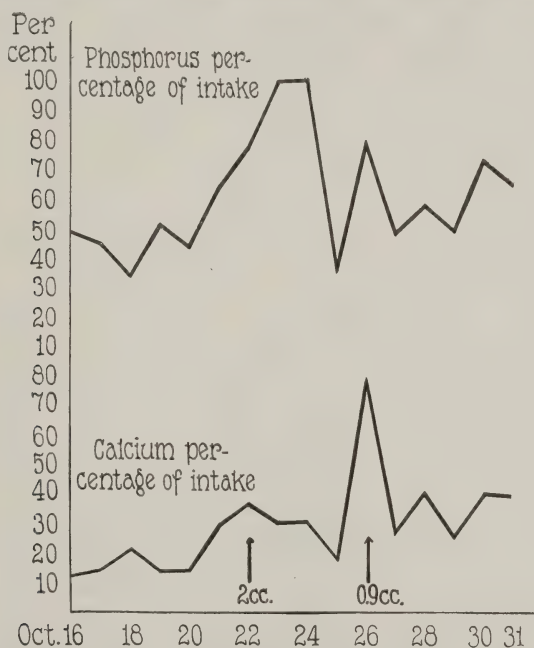


CHART 5. Urine analyses of calcium and phosphorus of Dog A on Diet A before and after oral administration of chaulmoogra oil.

extract as made upon dried material for 24 hours in the Soxhlet apparatus. Feces were marked off by adding to the food 2 gm. of carmine.

The female dogs were catheterized daily with sterilized instruments. In the later experiments when the hydnocarpate showed a marked effect on the ammonia excretion, the bladders were washed out daily with dilute permanganate solution. The spon-

taneously voided urine from the male dog was collected every 48 hours.

Diets.—The various diets consisted of lean meat, cracker meal, agar, and lard or butter in a suitable proportion. Table II shows the composition of the diets, with the analyses of their content of calcium, phosphorus, nitrogen, and fat.

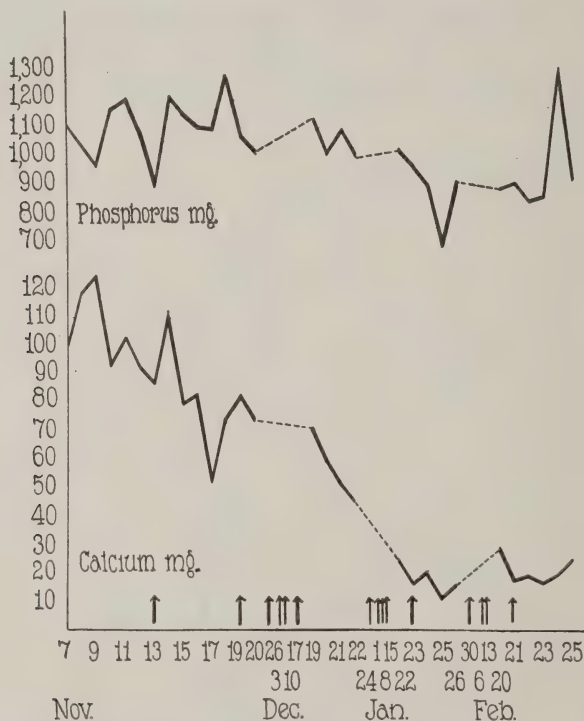


CHART 6. Urine analyses of calcium and phosphorus of Dog A on Standard Diet B after weekly subcutaneous injections of 0.5 cc. of ethyl hydrocarbate.

Dosage and Method of Administration of Chaulmoogra Oil.—Dog A after a fore period of 1 week was given 2 cc. of emulsified oil by stomach tube. There was emesis after 30 minutes. Extreme nausea persisted for 2 days. The appetite recovered fully on the 3rd day.

Administration of 0.9 cc. of oil in a formalized gelatin capsule on the 4th day caused emesis after 3 hours. The appetite was

TABLE III.
Average Daily Outputs and Balances of Dog A.

Weight 15.4 kilos.

Period No	Calcium.					Phosphorus.					Nitrogen.					Fat.			
	Dried feces.	Urine.	Feces.	Total output.	Intake.	Balance.	Urine.	Feces.	Total output.	Intake.	Balance.	Urine.	Feces.	Total output.	Intake.	Balance.	Percentage.	Daily output.	
	gm.	mg.	per cent	mg.	mg.	mg.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm.	gm.	gm.	
I	16.1	48	3.36	542	590	-341	1.14	3.12	0.51	1.65	-0.55	8.42	4.22	0.68	9.19	11.13	-1.94	5.46	0.88
II*	8.8	73	2.55	224	297	-104	1.14	2.22	0.20	1.34	-0.47	7.95	2.70	0.24	8.19	9.10	-0.91	2.69	0.24
III*	15.3	95	2.07	316	411	-218	1.05	1.98	0.30	1.35	-0.30	8.49	2.90	0.44	8.93	7.98	-0.95	2.90	0.44
IV†	12.1	78	2.20	267	345	-153	1.13	1.83	0.22	1.35	-0.18	7.72	1.95	0.24	7.96	7.62	-0.34	3.53	0.42
V†	14.5	56	2.51	364	420	-228	1.05	2.20	0.32	1.37	-0.17	7.85	2.65	0.39	8.24	7.89	-0.35	1.94	0.28
VI†	14.6	17	1.98	289	306	-145	0.90	2.22	0.32	1.22	-0.29	6.56	3.46	0.51	7.07	7.43	-0.36	3.60	0.53
VII†	18.8	21	1.53	288	309	-132	0.97	1.52	0.29	1.26	-0.45	7.01	2.80	0.53	7.54	8.09	-0.45	2.00	0.38

* Oral administration of large doses of oil (see Chart 5).

† Weekly subcutaneous injections of 0.5 cc. of ethyl hydnocarpate (see Chart 6).

unaffected, but subsequent attempts to feed the oil made the animal most fastidious, the slightest trace of oil creating nausea. After completing the month's analysis of urine and feces no further attempt was made to give the drug by mouth to this animal. Careful record was kept of the small variations in the intake of food, and the results were computed according to the percentage of the intake. Chart 5 shows how marked an effect was produced in increasing the relative urinary output of calcium. After complete rest Dog A was given weekly subcutaneous injections of

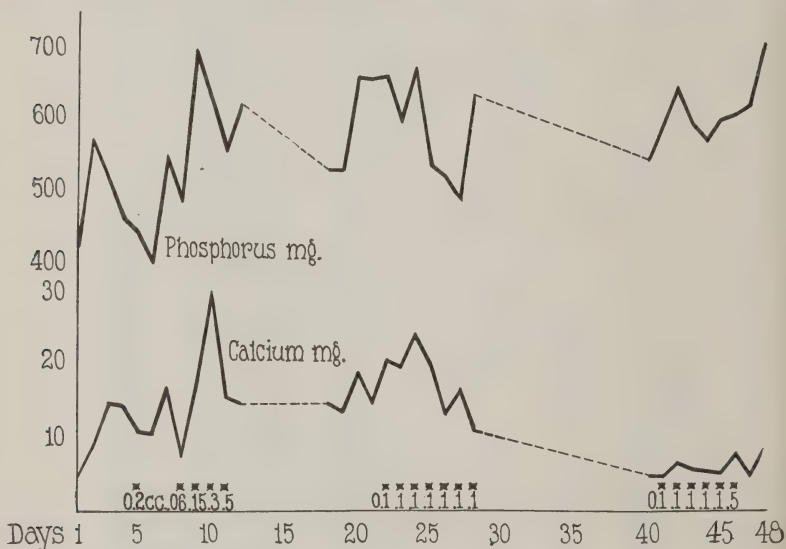


CHART 7. Urine analyses of calcium and phosphorus of Dog B on Standard Diet B. x oral administration of chaulmoogra oil.

ethyl hydnicarbate. The average balances and outputs are recorded in Table III and Chart 6.

A similar experiment was undertaken with Dog B. Instead of giving comparatively large doses of the oil a succession of smaller and less toxic doses, as indicated on Chart 7, was given. As soon as vomiting occurred, complete rest was allowed for several days, and then further analyses were undertaken. The excretion of calcium both in the urine and the feces was increased [after administration of the oil (Table IV, Periods I and II).

Later, Dog B was given an intraperitoneal injection of a large dose of the oil. The urinary excretion, see Chart 8, was not greatly affected, but the fecal output was greatly increased (Table IV). The total effect upon the calcium balance was similar to that obtained by oral administration.

This experiment was repeated on another animal, Dog C, with a result not greatly different from that obtained with Dog B.

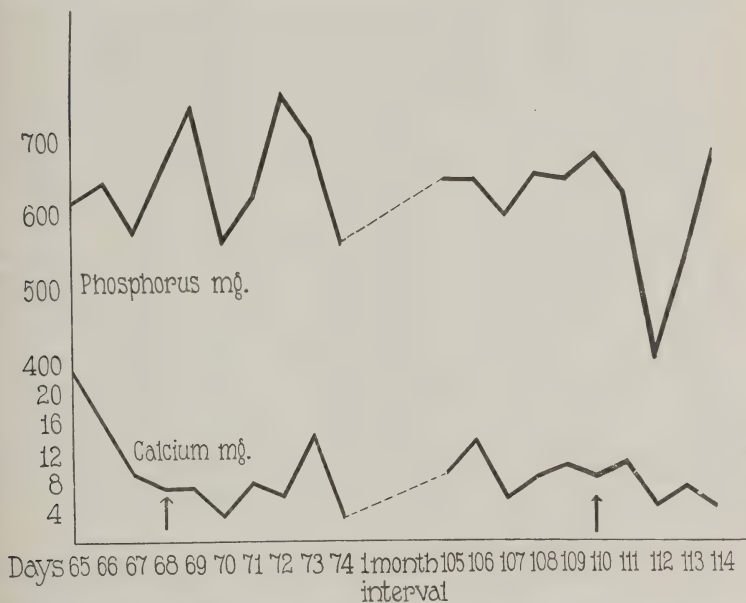


CHART 8. Urine analyses of calcium and phosphorus of Dog B on Standard Diet B after an intraperitoneal injection of 5 cc. of chaulmoogra oil.

Subcutaneous injection of 4 cc. of oil into Dog B produced an increase in the urinary calcium, but the fecal calcium was decidedly lowered, the total showing a retention of calcium (Table IV). This result probably represents the effect of long treatment, rather than what would be expected from the effect of a similar experiment upon an animal which had not already received many doses of the oil.

TABLE IV.
Average Daily Outputs and Balances.

Period No.	Remarks.	Calcium.					Phosphorus.					Nitrogen.					Fat.					
		Output.					Intake.					Output.					Intake.		Balance.	Percentage.	Daily output.	
		Urine.			Feces.		Total.	Urine.			Feces.		Total.	Urine.			Feces.					Total.
		gm.	mg.	per cent	mg.	mg.		gm.	mg.	per cent	gm.	mg.		gm.	mg.	per cent	gm.	mg.				
Oral administration. Dog B.																						
I	Normal.	8.2	11	1.67	137	148	115	-35	0.48	1.87	0.15	0.63	1.02	+0.39	3.60	3.83	0.31	3.91	4.83	+0.92	3.78	0.31
II	Oil by mouth.*	14.3	16	2.88	412	426	115	-311	0.56	1.49	0.21	0.77	1.02	+0.25	4.02	4.59	0.67	4.69	4.83	+0.14	5.67	0.81
III	Fore period.	10.4	16	2.31	239	255	115	-140	0.60	2.19	0.23	0.83	1.03	+0.20	4.20	4.44	0.44	4.64	5.06	+0.42	3.67	0.38
IV	Oil by mouth.*	10.0	17	1.55	155	172	115	-57	0.58	1.57	0.16	0.74	1.03	+0.29	3.62	3.45	0.34	3.96	5.06	+1.10	3.70	0.37
V	" "	8.9	6	1.34	119	125	102	-23	0.60	1.71	0.15	0.75	1.01	+0.26	4.40	4.54	0.40	4.80	5.23	+0.43	3.49	0.31
Before and after intraperitoneal injection. Dog B.																						
VI	Fore period.	9.0	12	1.39	125	137	115	-22	0.65	1.47	0.13	0.78	1.03	+0.25	4.33	3.65	0.33	4.66	5.06	+0.40	3.64	0.33
VII	After 5 cc. oil.	11.4	7	1.56	178	185	115	-70	0.64	1.48	0.17	0.81	1.03	+0.22	4.16	4.61	0.52	4.67	5.06	+0.39	3.34	0.38
VIII	Fore period.	11.2	9	1.28	144	153	100	-53	0.64	2.30	0.16	0.80	1.01	+0.21	4.01	3.99	0.45	4.46	4.98	+0.52	1.98	0.22
IX	After 5 cc. oil.	11.3	8	0.91	103	111	100	-11	0.59	2.00	0.15	0.74	1.01	+0.27	4.43	4.22	0.47	4.90	4.98	+0.08	4.36	0.48
Subcutaneous injection. Dog B.																						
X	Fore period.	9.4	6	1.38	130	136	102	-34	0.59	1.51	0.14	0.73	1.01	+0.28	4.35	3.83	0.36	4.71	5.23	+0.52	3.29	0.31
XI	After 4 cc. oil.	10.4	13	1.10	114	127	102	-25	0.63	1.30	0.14	0.77	1.01	+0.24	4.71	4.03	0.42	5.13	5.23	+0.10	3.84	0.40

Intraperitoneal injection with calcium-rich diet. Dog B.

XII	Fore period.	9.4	22	2.53	237	259	279	+20	1.86	0.17					3.86	0.36			2.62	0.25
XIII	After 8 cc. oil.	12.8	18	2.32	296	314	279	-35	1.98	0.25					4.42	0.56			3.28	0.42

Intraperitoneal injection. Dog C.

I	Fore period.	19.6	49	2.12	416	465	177	-288	1.09	2.23	0.44	1.53	1.71	+0.18	7.65	3.64	0.71	8.36	8.09	-0.25	3.34	0.66
II	After 5 cc. oil.	23.1	38	2.06	476	514	177	-337	1.02	2.14	0.49	1.51	1.71	+0.20	7.68	4.36	1.01	8.69	8.09	-0.60	2.44	0.57

*For dosage see Chart 7.

Volume and Composition of the Feces.

It should be noted that chaulmoogra oil increases peristalsis so that a greater volume of feces is obtained (compare Periods I and II, and Periods VI and VII, Dog B, Table IV; also Periods I and II, Dog C). An experiment, not reported here in detail, which terminated abruptly in an abortion, showed an increase from a daily excretion of 16.8 (dried feces) to 20.2 gm.

The increase in the volume of the feces would naturally make the output of calcium larger. In considering the results obtained, it is of importance to observe the percentage increase or decrease. Dog A, which vomited the oil, showed a relative and an absolute decrease after the first oral dose. Dog B, which experienced no such gastric disturbance, showed both a relative and an absolute increase in the amount of calcium excreted after the first oral administration of the oil. Later, the large intraperitoneal injection, Period VII, showed the same effect.

The dried feces in both the dog and rabbit experiments were extracted with chloroform, and with ether. The solutions obtained were carefully examined for the presence of rotatory oil. In no case did any extract from the feces rotate the plane of polarized light.

The development of a tolerance to the gastric effect of the oil is made the basis of treatment by gradually increased dosage from a small initial dose. The results here given are evidence for the development of a tolerance to the effect of the drug upon peristalsis and a hyperexcretion of calcium.

Dog B, Table IV, after continued oral administration, did not excrete such large volumes of feces. Period V is practically the same as the normal. A second intraperitoneal injection made in Period IX did not increase the bulk of the feces.

It is of interest to note that in these and the rabbit experiments, chaulmoogra oil produces an initial increase in peristalsis, but *it does not cause diarrhea*.

The effect upon calcium balance was not dependent on changes in the volume of the feces. The percentage output of Dog B shows a large initial increase after oral and peritoneal administration. Repeated dosage caused an opposite effect. Compare Periods III and IV, and Periods VIII and IX, Dog B, Table IV.

Toxic Effects Shown by Chaulmoogra Oil and Its Preparations.—From the outset it was obvious that in attempting to administer doses of this drug larger than the usual therapeutic amount, toxic effects were manifest which might seriously complicate the results of a chemical investigation.

The minor effect of nausea with vomiting and anorexia is readily observed. Likewise, the local irritant effect, which produces abscesses or local edema, has been demonstrated after the administration of large doses.

An extensive series of experiments was conducted, in which observation was made of the toxicity of chaulmoogra oil. These experiments are reported in another paper (8). The size of the dose and the path of administration may vary the toxic effect upon the liver, the kidneys, the blood elements, and the alimentary tract. There is also an action upon the central nervous system, and other minor effects which should not be overlooked.

Calcium Excretion after Intraperitoneal Injection of Chaulmoogra Oil with a Calcium-Rich Diet.

Experiment 1.—Dog B after a 10 day rest was used for a further trial of the effect of the oil on calcium metabolism when the diet contained much of this element. 7 gm. of calcium lactate (= 1.25 gm. of CaO) were added once to the food during each of the 2 weeks before and after intraperitoneal injection of 5 cc. of oil. The general effects were similar to those in previous experiments. The urinary excretion was decreased and the fecal greatly increased, the total negative balance being much increased. The weekly totals were: before, 1.815 gm.; and after administration, 2.199 gm. (see Periods XII and XIII in Table IV).

It should be noted that the fore period shows a positive daily balance of + 20 mg., but after the oil there was a negative balance of -35 mg. This confirms the previous results of intraperitoneal injection of large doses of the oil.

Experiment 2.—Using Dog C, an attempt was made to repeat the last experiment. 10 cc. were given intraperitoneally. Death unexpectedly resulted about 3 hours later.

C. Calcium Deposition in Mice after Administration of Ethyl Hydnocarpate.

Miss Hartley C. Embrey, Food Chemist to the Peking Union Medical College, in a private communication, stated that the addition of the mixed ethyl esters to the diet of mice produced

increased deposition of bone calcium. It seemed probable that injection might have the same effect.

To test this hypothesis mice were maintained under the following experimental conditions.

Two litters of mice were placed on a mixed diet. In each litter one animal served as a control, the second received a subcutane-

TABLE V.
Calcium Deposition in Mice. Body Weight and Calcium in Ash.

Week.	Remarks.	Weight.							
		Litter 1.			Litter 2.			Litter 3.	
		Ethyl hydnocarpate, 0.1 cc. each dose.			Crude ethyl hydnocarpate, 0.05 cc. each dose.			Ethyl hydnocarpate, 0.1 cc. each dose.	
		Control.	Subcutaneous.	Intraperitoneal.	Control.	Subcutaneous.	Intraperitoneal.	Control.	Subcutaneous.
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	Injection.	17.0	19.0	16.5	9.5	9.5	12.0	14.25	16.0
2	"		19.0		11.5	12.5	13.0	17.30	18.0
3	"		19.0	19.5	14.25	13.5	14.5	17.50	18.5
4	"	20.0	19.0	23.5	15.0	16.3	18.5	18.30	19.25
5	No injection.	23.5	25.0	24.8	19.3	18.3	21.0		
6	" "	24.5		27.0		20.5	24.5	23.25	23.25
7	" "	24.5	25.5	27.3					
8	" "		26.5		21.3	22.75	26.75		
9	" "	29.0	26.8	29.0					
Calcium oxide in total ash of body, mg.		284	299	272	244	253	256	204	213

ous injection, and the third, an intraperitoneal injection. The animals in the first litter were given ethyl hydnocarpate, those in the second litter, crude ethyl hydnocarpate.

A third litter of two mice was maintained upon a plain oat diet. One served as a control, the other received a subcutaneous injection of ethyl hydnocarpate.

The results summarized in Table V fail to show sufficiently great divergencies from the normal to lead to positive conclusions. It is quite possible that the negative result may have been due to

the fact that too large doses of the drug were administered. This view-point is supported by the fact that the appetite was seriously affected at the beginning. As soon as the injections were stopped in Lot 2, the treated mice gained in weight more rapidly than the control.

TABLE VI.

Estimation of Blood Calcium after Administration with the Hydnocarpates.

Experiment No.	Method of administration.	Calcium per 100 cc. of serum.	Remarks.
		mg.	
1	Intraperitoneal injection.		
	Dog B.		
	Before; normal.	10.0	
	After; 5 cc. of oil.	11.9	
	Dog C.		
	Before; normal.	11.7	
	After.	12.1	
2	Weekly subcutaneous injection of 0.5 cc. of ethyl hydnocarpate.		
	Dog A.		
	Mar. 3. Before.	11.7	
	After.	11.8	
	Mar. 14. Before.	11.4	
	After.	11.2	
3	Oral administration of a fatal dose of oil.		
	Rabbit 17.		
	Before.	10.6	
	After.	10.0	
	Rabbit 1.		
	Before.	8.6	
	After.	7.0	Tetany.
4	Intraperitoneal injection of fatal dose (after many injections).		
	Rabbit 3.		
	Before.	7.9	
	After.	7.4	

In Lot 3 the treated mouse had a ravenous appetite and was quite lively, but it did not put on as much weight as the control which had a very small appetite.

It is quite evident, therefore, before this point can be established that further investigation will be necessary.

D. Estimations of Blood Calcium after the Administration of the Hydnocarpates.

With such a marked effect upon the calcium metabolism, it might be expected that hydnocarpus oil would alter the calcium content of the blood. A number of determinations were made by Tisdall's method (9), the results of which are recorded in Table VI.

In two dogs, Dogs B and C, after intraperitoneal injection, there was an increase in blood calcium. Dog A, which had been receiving weekly injections of ethyl hydnocarpate for 4 months, gave the value 11.7 mg., which is within the range of normal content. This value was maintained with slight variation during the 12 days that the injections were continued and the estimations made.

The three rabbits to which fatal doses were administered showed a decrease in the blood calcium. In one case this was associated with tetany. This is discussed more fully in a separate publication upon the toxicology of chaulmoogra oil (8).

Rabbits 1 and 3, after treatment with toxic doses of the oil, showed low values for blood calcium when compared with the normal figures given by Denis (10). The further reduction of these values was associated with conditions which resulted fatally.

Relationship of Phosphorus and Fat Metabolism to Calcium Excretion after Administration of the Hydnocarpates.

A. Urinary Phosphorus Excretion.

It is significant that there is no real parallel established between the urinary excretion of phosphorus and calcium after the administration of the hydnocarpates in a variety of ways and in large and small doses. Where such a parallel appears to exist there is a more striking relationship between the phosphorus and the nitrogen excretion. This is noticeably so in the urine analyses of Dog A on Oct. 19 and 25, Nov. 10 and 19, Dec. 21, Jan. 24, and Feb. 22 and 25 (Charts 5 and 6). The same observation was made concerning the results from Dog B.

In the rabbit experiments, identical results were obtained in even a more clear-cut way. In Experiment 1, after the oral administration of 5 cc. of

oil, the calcium excretion rose from 10.8 to 22.7 mg. The phosphates fell from 0.700 to 0.599 gm., which corresponds with the decrease in nitrogen (see Chart 1).

After subcutaneous injection of the oil the increased calcium output does not accompany a corresponding increase in the phosphate excretion. On the day subsequent to both the first and third injections, the phosphates follow the nitrogen and not the calcium. The same is true for this animal after both intraperitoneal injections.

The very significant output of calcium after the intravenous injection of ethyl hydnocarpate, Rabbit 12, Chart 4, was unattended by an increase in the phosphate excretion, and the subsequent fluctuations in the amounts of calcium excreted in the urine are without parallel in the determinations for phosphate.

The experiment with the subcutaneous injection of ethyl hydnocarpate, Rabbit 13, Chart 3, brings out a novel feature. On the 3rd, 4th, and 5th days subsequent to the first injection, the phosphate excretion is not parallel to the calcium, nor is it apparently related to the nitrogen output. Where there is no change in the acidity to account for a difference in phosphate excretion, and where the phosphate does not run parallel to the calcium and nitrogen, its variation seems to follow a similar alteration in the amount of calcium excreted on the previous day. In other words, there is apparent a phosphate "lag." This is strikingly noticeable in Rabbit 13, on the 14th, 15th, 16th, and 21st days; in Rabbit 11, on the 16th, 28th, and 34th days; and in Rabbit 14, on the 11th day. (See Chart 2.) There were so many factors influencing the excretion of the phosphates that a similar relationship between calcium and phosphates for determinations made on other days of the experiments is rendered less clear.

B. Comparative Elimination of Phosphorus and Calcium in the Feces.

After the oral administration of chaulmoogra oil, both in large doses to Dog A and in small doses to Dog B, the excretions of phosphorus and calcium in the feces are parallel in character. A decrease in calcium is associated with a similar decrease in phosphorus, and the rule holds true for the periods when an increase in either element occurs (see the average daily excretions for Dog A, Table III, and for Dog B, Table IV).

When large doses were administered intraperitoneally, there was likewise a parallel relationship shown between the amounts of phosphorus and calcium excreted daily. This ratio does not apply in all cases if only the percentage content of the elements is considered, because the volume of the feces shows a decided variation before and after treatment, and after repeated treatment. The irritant character of the oil increases peristalsis. In some of the rabbit experiments when the drug was introduced intravenously or subcutaneously, there was no diarrhea, but the feces were softer than normally, and Dog A, after 5 months of weekly subcutaneous treatment, showed signs of a cumulative effect in producing vomiting and a

much greater weight of feces. This indicates the possibility of there being more than a local irritant effect. From the foregoing facts, it is probable that the calcium is excreted in the feces as phosphate.

C. Phosphorus Balance.

In all of the experiments there was a positive phosphorus balance, the figures failing to show any significant differences. There does not appear to be any consistent ratio between the balances of calcium and phosphorus (Tables III and IV).

D. Ratio between Calcium and Fat.

Elimination of calcium and fat approached a parallel during the periods of oral administration of chaulmoogra oil. In Dog A, after the first subcutaneous injection of ethyl hydnocarpate, this direct ratio was undisturbed. However, during the remainder of the treatment no such parallel relationship was maintained between the fecal excretion of fat and calcium. On Nov. 23, 50 gm. of butter were added to the diet in place of 20 gm. of lard. The results in Table III show that calcium and phosphate in Period V returned to the higher level of Period II, but the percentage of fat was the lowest obtained in any period of the experiment.

Early in January, the fourth month of the experiment, owing to lack of appetite, the amount of butter was reduced to 10 gm. The animal was in excellent condition and did not appear to need the extra calories. Subsequent to this change, there was a large increase in the fat excretion, which was apparently unrelated to the decreased calcium output. A month later the calcium showed no change, but the fat percentage and total output were decidedly less.

The parallel excretion of calcium and fat in Dog B after the oral administration of the oil was not maintained in the subsequent experiments. Consideration of the results obtained from Dogs B and C, after intraperitoneal injection, established the fact that there is a marked inverse ratio between the percentages of calcium and fat excreted in the feces in Dog B, Periods VI to XIII. Dog C shows this inverse ratio only between the average daily amounts, the percentages are in direct ratio. In this experiment with a male dog, the result differed from all of the other similar experiments in that, while the weight of feces was similarly increased, the total amount of fat present was less, whereas in the five other experiments there was an increase in the amount of feces (Table IV).

These observations lead to the conclusion that the normal parallelism between calcium and fat excretion is markedly disturbed by large doses of chaulmoogra oil. This may be partly due to the increased peristalsis induced by the local irritant character of the oil and by its cumulative effect upon the gastrointestinal tract through the nervous system.

DISCUSSION.

Underhill, Honeij, and Bogert (11) have shown that in two cases of leprosy there was a definite retention of calcium. In the more advanced stage of the disease the degree of retention was greater. This behavior was taken to indicate that the organism is in need of calcium, and they suggest the administration of calcium as an additional therapeutic measure. Bonlay and Leger (12, 13) later made studies of the calcium balance in four cases of leprosy. They found calcium retention in the two early mild cases and hyperexcretion in two advanced cases with loss of digits and necrosis of tissue of the feet and hands. They consider that the first two cases confirm the work of Underhill. Their deductions were dissimilar. They state that the two types correspond with the two different clinical states, the simple anesthetic type showing a retention of calcium greater than that of the normal subject. The calcium is excreted as phosphate, but in the advanced cases some other acid, probably organic, which causes hyperexcretion of calcium, is present in the system. This is based upon their analyses of phosphorus as well as calcium in the urine and feces.

If the observations of Bonlay and Leger are general for all advanced cases of leprosy associated with absorption of the bones, the results here reported on calcium metabolism may explain why the chaulmoogra oil treatment has been found of little benefit to such patients (14), while sodium morrhuate is distinctly useful (1, 3). Rogers has explained the action of chaulmoogra oil as being common to all the unsaturated fatty acids, and the effects of morrhuates, soyates, linoleates, etc., as being a matter of degree rather than of specificity of any of these oils. The present investigation fails to support this hypothesis, for the control experiments with olive oil showed no effect like that noted with chaulmoogra oil upon calcium metabolism.

There are some reactions of chaulmoogra oil which show similar or opposite character to these well known fatty acids. In a recent paper, Brinkman and von Szent-Györgyi (15) state: "The fatty acids present in normal blood or added purposely are combined as calcium soaps. Probably this calcium combination is the cause of the disappearance of the hemolytic power of stearic, palmitic and oleic acids in serum. On the other hand, the hemoly-

tic power of linoleic acid is greatly increased." In the experiments here reported when the glyceryl or ethyl esters were used, there was marked hemolysis with a hyperexcretion of calcium. It appears as if the esters were hydrolyzed by the body to form the free fatty acids, chaulmoogric and hydnocarpic acids, which are not resynthesized into fat, but act in a similar way to free fatty acids like oleic and linoleic acids when they are introduced in their free form into the system. The hyperexcretion of urinary calcium after the administration of chaulmoogra oil to rabbits produces an exactly opposite effect to that obtained by Sjollem (16) after the administration of cod liver oil.

Whether the retention of calcium in the cases of early leprosy is an indication of excess or of a need for that element in the body, further investigations will make clear. It is evident that chaulmoogra oil has the character of a transporter of calcium in the blood stream. The experiment upon Dog A shows that the prolonged ester treatment, found of curative value in leprosy, is conducive to the retention of calcium in the body. It is seen in a variety of the experiments reported that large sporadic doses remove calcium from the system. This fact indicates that there is possibly harm done by therapeutic treatment along such lines. Muir (17) warns against the bad effects of the sudden administration of large doses of the esters. This he has related to the febrile reactions produced. He recommends starting with a small dose and subsequently increasing the amount. At no time did he employ such large doses as were seen in early reports when the value of chaulmoogra oil as a leprosy specific was the subject of much controversy.

It is clear that there is need for a study of the calcium metabolism of leprosy patients under treatment with the hydnocarpates. The preliminary hyperexcretion of calcium, following the administration of the hydnocarpates, may be related to the striking effects seen in early anesthetic types, in which pigmentation and anesthesia have cleared up after three injections of the ethyl esters.

The repeated oral administration of the oil to Dog B showed that a tolerance to its original metabolic effects is produced and that the effects are similar to subcutaneous injections of the ethyl ester into Dog A in developing calcium retention.

CONCLUSIONS.

1. Chaulmoogra oil or ethyl hydnocarpate administered in various ways in large doses to rabbits produces a marked *increase in urinary calcium*.

2. Chaulmoogra oil given in small oral doses to dogs produces a marked increase in urinary calcium, and an increase in fecal calcium. Continued administration reverses this effect, and *favors calcium retention*.

Prolonged hypodermic administration of ethyl hydnocarpate in therapeutic doses to dogs decreases the urinary calcium and favors calcium retention. Large intraperitoneal injections decrease the urinary calcium and greatly increase fecal calcium so that there is a hyperexcretion of calcium.

3. Analyses of the phosphorus and fat show that they bear little relationship to the changes in calcium metabolism. Calcium is excreted in some form other than the urinary phosphates or the fecal soaps found in the normal subject.

4. There is observed an increase in the calcium of the blood after the administration of the hydnocarpace to dogs. Large doses administered to rabbits, producing a fatal effect, cause a decrease in blood calcium with tetany.

5. The chemical analyses made show that there is the development of a tolerance to the original effect of the drug upon metabolism, particularly as seen in the lessened local irritant effect upon the alimentary canal.

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METABOLISM STUDIES WITH CHAULMOOGRA OIL.

II. THE INFLUENCE OF THE HYDNOCARPATES UPON URINARY NITROGEN PARTITION IN THE DOG.*

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In the metabolism studies made with chaulmoogra oil, there has been shown an increase in the output of urinary calcium associated with a rise in the nitrogen excretion. Concurrent and subsequent to the calcium studies, experiments were undertaken to determine the character of this increased nitrogen output.

The existing theories concerning the effects of chaulmoogra oil, obtained after its administration to lepers, are for the most part not based upon laboratory findings. Further chemical study may bring evidence to support the theory of "leucocytosis" of Talwik (1), or the "organic acid leucocytic hypergenesis" theory of Mercado (2). It may be possible to make some deduction concerning the nature of the "gloea substrate" which according to Muir (3) holds the leprosy bacilli embedded in the lymph spaces.

EXPERIMENTAL.

The standard conditions prevailing in the experiments upon calcium metabolism were maintained. The urines were collected by careful catheterization of the female dogs. When the ammonia values were observed to rise, the bladders were washed out daily with dilute permanganate. There was no evidence of infection. To confirm the results obtained, a male dog was used, and the spontaneously voided urines were collected and showed a similar

* The data are taken from the thesis of Bernard E. Read presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1924.

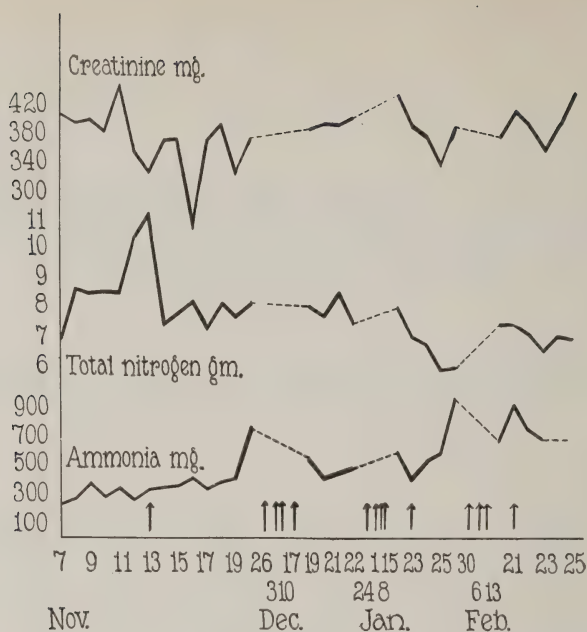


CHART 1. Urine analyses of nitrogen, ammonia, and creatinine of Dog A on Standard Diet B after weekly subcutaneous injections of 0.5 cc. of ethyl hydnocarpate.

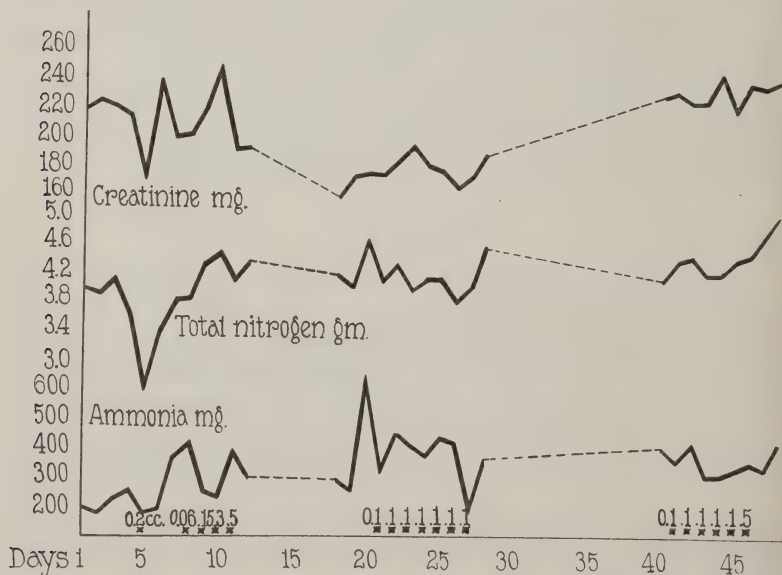


CHART 2. Urine analyses of nitrogen, ammonia, and creatinine of Dog B on Standard Diet B. * oral administration of chaulmoogra oil.

change in the ammonia excretion after the administration of chaulmoogra oil.

Analyses were made for ammonia and creatinine by Folin's aeration and colorimetric methods (4). Samples of urine were titrated with 0.1 N sodium hydroxide for acidity values.

The results summarized in Tables I, II, and III show the average daily outputs during the several periods of analyses of the

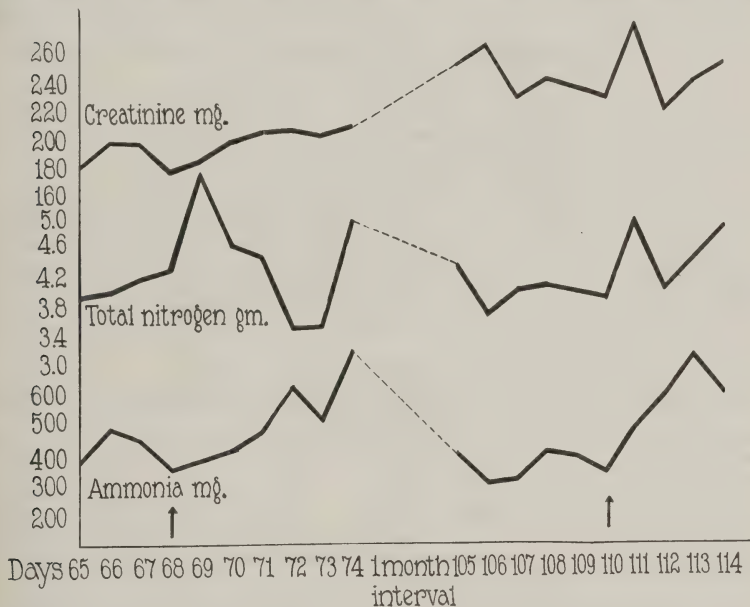


CHART 3. Urine analyses of nitrogen, ammonia, and creatinine of Dog B on Standard Diet B after intraperitoneal injections of 5 cc. of chaulmoogra oil.

urines of 2 female and 1 male dog. Charts 1, 2, and 3 show the immediate effects in detail, the immediate as distinguishable from the delayed or after effect of the oil.

1. Total Nitrogen Excretion.

The results show that there is a definite increase in the amount of nitrogen excreted in the urine after the oral administration of chaulmoogra oil (Table II and Chart 2). After intraperitoneal

TABLE I.

Urine Analyses of Dog A. Daily Averages before and after Treatment.

Date.	No. of days.	Remarks.	Titratable acidity.	Nitrogen.		Ammonia.		Creatinine.	
			cc. 0.1 N NaOH	gm.	per cent of in- take	gm.	per cent of total N	mg.	per cent of total N
Nov. 6-13	8	Fore period.	151.0	8.49	115.8	0.292	2.84	374	2.88
" 13		0.5 cc. ethyl hydno- carbate, subcu- taneously, weekly injections con- tinued up to							
" 14-20	7	Feb. 26.	165.7	7.72	100.7	0.426	4.54	348	2.94
Dec. 19-22	4		113.9	7.85	99.6	0.468	4.92	386	3.21
Jan. 22-26	5	" "	91.2	6.56	88.4	0.608	7.64	379	3.77
Feb. 20-25	6	" "	40.5	7.01	86.7	0.887	10.04	391	3.64

TABLE II.

Urine Analyses of Dog B. Daily Averages. Oral Administration of Chaulmoogra Oil.

Period No.	No. of days.	Remarks.	Titratable acidity.	Total N.		Percentage of total N.	Creatinine.	Percentage of total N.
			cc. 0.1 N NaOH	gm.	NH ₃ gm.	per cent	mg.	per cent
I	5	0.2 cc. oil given on 5th day.	52.3	3.60	0.204	4.67	209	2.16
II	7	0.6, 0.15, 0.3, 0.5 cc. oil given on 3rd, 4th, 5th, and 6th days, respectively.	55.4	4.02	0.332	6.79	212	1.96
		5 day interval.						
III	5	Fore period.	64.8	4.20	0.383	7.52	178	1.58
IV	6	0.1 cc. of oil given daily.	65.9	4.08	0.361	7.28	179	1.63
		12 day interval.						
V	9	0.1 cc. daily on 5 days then 0.5 cc. on 7th day.	44.7	4.40	0.354	6.64	232	1.96

administration there is also an immediate increase in the nitrogen output (Chart 3), but the subsequent effect is to delay the

nitrogen excretion so that the total amount excreted in a given period after giving the oil is practically the same as that in the fore period (Table III).

In the case of Dog A treated with weekly injections of ethyl hydnocarpate in the course of 4 months there was a progressive decrease in the urinary nitrogen excretion (Table I). In the previous paper there was reported the fecal excretion, which increased from 0.237 to 0.527 gm. This was not coincident with the changes

TABLE III.
Urine Analyses, Daily Averages.

Period No.	No. of days.	Remarks.	Acidity.	Total N.	Ammonia.	Percentage of total N.	Creatinine.	Percentage of total N.
Dog B. Intraperitoneal injection of 5 cc. of chaulmoogra oil.								
			cc. 0.1N NaOH	gm.	gm.	per cent	mg.	per cent
VI	5	Before.	69.0	4.33	0.405	7.71	189	1.62
VII	5	After.	61.5	4.16	0.563	11.13	205	1.83
VIII	5	Before.	46.8	4.01	0.340	6.98	244	2.26
IX	5	After.	22.8	4.42	0.554	10.32	243	2.04
Dog B. Diet B. Subcutaneous injection of 4 cc. of chaulmoogra oil.								
X	5	Before.	47.4	4.35	0.322	6.09	232	1.98
XI	5	After.	27.3	4.71	0.518	9.04	250	1.97
Dog C. Diet B. Intraperitoneal injection of 8 cc. of chaulmoogra oil.								
I	8	Before.	186.0	7.65	0.378	4.07	344	1.67
II	8	After.	181.0	7.68	0.601	6.45	361	1.75

in the percentage content of the feces, but was related to a certain looseness of the bowels occurring after prolonged treatment.

It is noteworthy that these results with dogs are different from those with rabbits reported in the paper upon calcium metabolism. In a number of experiments upon rabbits, irrespective of the path of administration, there was always a large increase in the urinary nitrogen after large doses of chaulmoogra oil or ethyl hydnocarpate. This result was obtained even when anorexia persisted for a number of days, thus indicating a considerable degree of tissue breakdown.

2. *Ammonia Excretion.*

One of the most striking effects upon the first dog treated was to produce an increase in the excretion of ammonia (Table I). A survey of this and of the urine analyses of Dog B (Tables II and III) shows that, after the continued administration of chaulmoogra oil by mouth and intraperitoneally or ethyl hydnocarpate subcutaneously, the percentage of ammonia nitrogen rises to a quite abnormal figure.

Dog B had a normal average daily output of 4.67 per cent of ammonia nitrogen in the urine. After small oral doses of oil, this figure increased to an average of 6.64 and 7.52 per cent in Periods II and III respectively (Table II). The subcutaneous injection in Period X brought it up to 9.04 per cent. In Period VI, after the intraperitoneal injection, it reached an average of 11.13 per cent. This was associated with a change in the acidity. The urine did not give an alkaline reaction to phenolphthalein until after the second large intraperitoneal injection, when 12.1 per cent of ammonia nitrogen was excreted (see Chart 3).

A male dog, Dog C, similarly treated with chaulmoogra oil gave a normal average daily excretion of 4.07 per cent of ammonia nitrogen. After an intraperitoneal injection of 8 cc. of oil, there was a progressive increase in the excretion of ammonia from 5.31 per cent on the 1st day to 7.73 per cent on the 3rd day. There was an average daily excretion of 6.45 per cent during the 8 days following the injection. This experiment when compared with the experiments upon Dog B yielded comparable results from the viewpoint of time intervals. Further comparison was prevented by the death of the animal following a large dose of the oil.

Hence one may conclude that chaulmoogra oil has an influence in decidedly increasing the urinary excretion of ammonia.

3. *Preformed Creatinine Excretion.*

A. Oral Administration of Therapeutic Doses.—After the oral administration of small doses of chaulmoogra oil, there was a noticeable change in the preformed creatinine values. In Dog B, the average normal excretion of creatinine for 4 days was 219 mg., 2.13 per cent of the total nitrogen. This normal excretion was disturbed by an oral dose of 0.2 cc. of oil. Further irregularity was produced by the doses of oil given on the 8th and subsequent days (Chart 2).

Two types of effect upon creatinine excretion may be seen after the administration of chaulmoogra oil. At first there is an immediate increase associated with an augmented nitrogen elimination. This is probably indicative of tissue breakdown. The highest figure in Period II occurs on the 10th day; namely, 248 mg. While this is decidedly greater than the normal, expressed as a percentage of the total nitrogen, 2.08 per cent, it is slightly lower than the normal, 2.16 per cent.

Following treatment there occurs a subnormal excretion of creatinine. After the second period this reached the low level of 159 mg., 1.43 per cent (Period III, 18th day, Chart 3). The amount gradually increased until the administration of oil again commenced when there was observed a sudden large output, namely 193 mg., the average elimination of the fore period being 178 mg.

After a 12 day interval, there was obtained on the 40th day of the experiment 228 mg. of creatinine. This high value coincided with a high nitrogen excretion. Oil was administered throughout this period. While there was maintained the high average of 232 mg. with a maximum of 244 on the 44th day, it represented only 1.96 per cent of the nitrogen excreted. This is a further demonstration of the early effect produced by the oral administration of chaulmoogra oil.

The second type of effect, the later pronounced reduction in the output of preformed creatinine, is probably associated with the temporary anemia, observed in rabbits after the administration of chaulmoogra oil.

B. Subcutaneous Injection of the Oil.—Similar results were observed in Dog B after the subcutaneous injection of 5 cc. of oil. There was an immediate hyperexcretion of creatinine associated with an abnormally high output of nitrogen. The values obtained in Periods X and XI, Table III, daily nitrogen excretion of 4.71 gm. as compared with 3.60 gm., and the average daily creatinine excretion of 250 mg. as compared with 209 mg., show that although there was a big increase in output from 209 to 250 mg. of creatinine, there was no appreciable change in the creatinine percentage of total nitrogen, which was indicative of considerable tissue breakdown.

C. Intraperitoneal Injection of the Oil.—Intraperitoneal injection of the oil in Dog B produced a large absolute and relative increase in the creatinine excretion (Periods VI and VII, Table III). A second similar injection caused the highest excretion obtained at any time with this animal, 270 mg. While the average output in Periods VIII and IX shows little change, the percentage is decreased from 2.26 to 2.04.

Dog C after similar treatment gave the same results. The average daily normal excretion of 344 mg. of creatinine was increased to 395 mg. on the first 2 days after the injection. The average daily percentage of creatinine nitrogen (1.67) was increased to 1.86 on the 3rd and 4th days.

D. Subcutaneous Injection of Ethyl Hydnocarpate.—The results from Dog A suggest similar effects to those reported after administration of the oil to Dog B, yet the immediate hyperexcretion associated with high nitrogen output is not so pronounced, and the later periods do not show it at all. There is a very marked low value on the 2nd or 3rd day after the injections, which is associated with a low value for nitrogen. The creatinine later increases while the total nitrogen is decreased or remains stationary. Creatinine reaches a maximum on the 4th or 5th day after injection.

It is of interest to note that where a high creatinine value is associated with a low total nitrogen excretion, it is often accompanied by a large amount of ammonia (Dec. 22, Jan. 22 and 26, and Feb. 21 and 25, Chart 1). The significance of this fact is not clear, although one may infer that it is associated with acid excretion.

TABLE IV.

Total and Preformed Creatinine before and after Administration of the Hydnocarpates.

Remarks.	Preformed creatinine.	Creatinine.	Total creatinine.	Preformed creatinine Total creatinine
Dog A. Continued weekly injections of ethyl hydnocarpate.				
Feb. 20	374	367	741	<i>per cent</i> 50.4
" 21 Injection of 0.5 cc.	407	448	855	47.6
" 22	391	328	719	54.4
" 23	355	324	679	52.3
" 24	388	290	678	57.2
" 25	431	410	841	51.2
Dog C, male. Intraperitoneal injection of chaulmoogra oil.				
48 hr. periods spontaneously voided urines.	660	1,091	1,751	37.7
	847	1,248	2,095	40.4
	731	934	1,665	43.9
	512	788	1,300	39.4
Normal average.....	688	1,014	1,702	40.4
After 8 cc. oil intraperitoneally.	789	901	1,690	46.7
	742	1,121	1,863	39.9
	674	636	1,310	51.4
	682	796	1,478	46.2
Average.....	722	864	1,586	45.6
Dog B. After second intraperitoneal injection.				
Jan. 24	229	253	482	47.5
" 26	235	263	498	47.1
Average.....	232	258	490	47.3
Jan. 28 Injection of 5 cc. oil.				
" 29	270	346	616	43.8
" 30	222	277	499	44.4
" 31	242	239	481	50.3
Feb. 1	254	209	463	54.9
Average.....	247	268	515	47.9

4. *Creatine Excretion.*

The variation in the excretion of creatinine suggested that possibly the creatinine excretion would show similar changes. A number of determinations were made upon Dogs A, B, and C. The results (Table IV) are of interest in that the creatine excretion does not follow that of creatinine, nor does the total creatinine remain stationary.

There is no consistent parallel between any two of the experiments, perhaps owing to the fact that the experimental conditions were not the same in the different experiments. With further work it may be possible to give a clear interpretation of these results.

DISCUSSION.

The introduction of chaulmoogra oil into occidental therapeutics has met with varied success. Efforts were made from almost the very first trials to overcome its unpleasant toxic effects. Given in large doses by mouth, there is produced acute nausea and anorexia, and subcutaneously the local irritant effects have caused much trouble. The experiments here reported show that large doses of chaulmoogra oil, previously used in therapeutics, produce a marked effect on the nitrogen metabolism. The increased urinary nitrogen is indicative of tissue breakdown, which may be associated with other effects noted (5). A febrile reaction is usually associated with increased nitrogen metabolism, and where there is a definite destruction of blood cells, there should be a change in the excretions. The high creatinine values obtained in Dog A on the 4th or 5th day after subcutaneous injection of ethyl hydnocarpate coincided with low hemoglobin values.

Wallace and Pellini (6) have shown that substances which have a widespread poisonous action on the capillaries produce a marked degree of acidosis. They assume that this is due to a state of suboxidation in the tissues. Chaulmoogra oil showed a decided effect upon the blood vessels of the ears of rabbits when there was seen dilation of the vessels, edema of the surrounding tissue, and bleeding from the inside of the ear. The ears were noticeably hot, irrespective of whether there was a general febrile reaction. These observations together with the high urinary ammonia values, and the subsequently lowered creatinine values, led to the supposition that the tissue metabolism is disturbed, chaulmoogra oil acting as a capillary poison. When the system is supplied with a quantity greater than it can dispose of, there is a condition of suboxidation in the tissues.

CONCLUSIONS.

1. There is an increase in the amount of nitrogen excreted in the urine after administering the hydnocarpates (chaulmoogra oil or the ethyl ester).

2. Continued administration of small doses of ethyl hydnocarpate causes an acidosis with suboxidation in the tissues as evidenced by a progressively decreasing urinary nitrogen and a large increase in the ammonia excretion.

3. Chaulmoogra oil in large doses intraperitoneally, and small doses orally, causes a decided increase in the ammonia excretion.

4. There is an immediate increase in creatinine excretion after administration of chaulmoogra oil associated with a high output of nitrogen in the urine.

5. High creatinine excretion occurring with a low total nitrogen output in the urine was found to be associated with high ammonia values, indicative of a greater excretion of acid after the administration of chaulmoogra oil.

6. A later effect of chaulmoogra oil is to cause a subnormal output of creatinine in the urine, probably related to a temporary anemia.

7. There is produced a marked increase in the ratio between preformed and total creatinine after administration of the hydnocarpate. The creatine values show considerable variation, which indicates that there is considerable disturbance in the regular metabolic processes.

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EXPERIMENTS ON THE WALDEN INVERSION.

PART XI.

SUBSTITUTION BY HALOGEN OF THE HYDROXYL GROUP IN THE SECONDARY OCTYL ALCOHOLS.

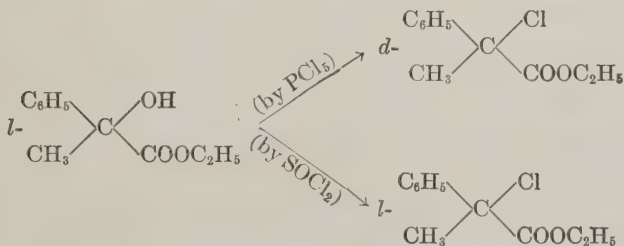
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TUDHOPE.

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(Received for publication, October 4, 1924.)

Our attention has just been directed to a recent paper by Levene and Mikeska¹ in which the secondary octyl alcohols are considered in their relationship to the Walden inversion. As we have also been engaged in this subject, we think it desirable to place on record an account of a few experiments in the same direction.

In the course of previous work^{2,3} the difference between the action of phosphorus pentachloride (or trichloride) and of thionyl chloride on optically active hydroxylic compounds has been observed on several occasions, thus:



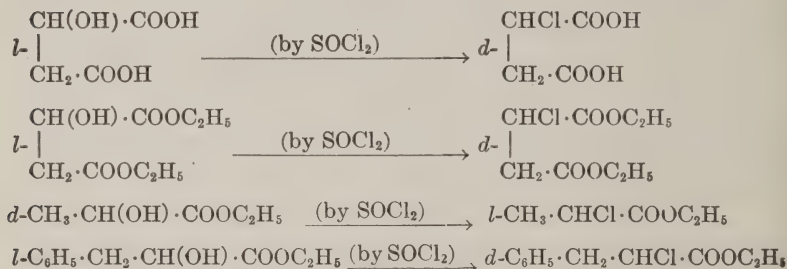
¹ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473.

² McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1908, xciii, 811; 1909, xcv, 777; 1910, xcvii, 1016, 2564; 1912, ci, 390. McKenzie, A., and Humphries, H. B. P., *J. Chem. Soc.*, 1910, xcvii, 121. McKenzie, A., and Wren, H., *J. Chem. Soc.*, 1910, xcvii, 1355. McKenzie, A., and Barrow, F., *J. Chem. Soc.*, 1911, xcix, 1910. McKenzie, A., and Walker, N., *J. Chem. Soc.*, 1915, cviii, 1685.

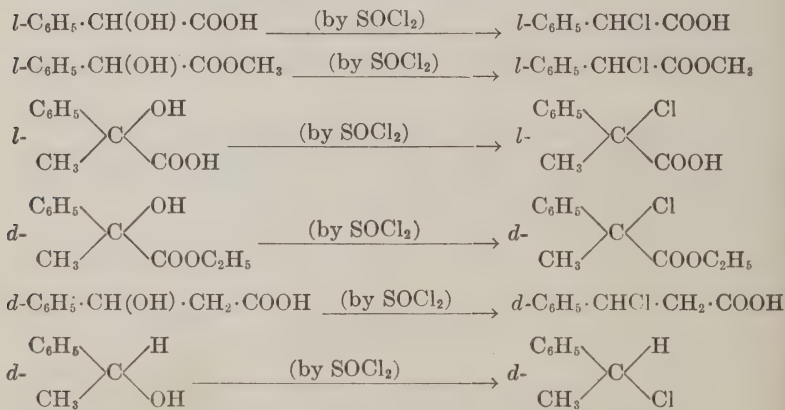
³ McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1913, ciii, 687.

While the action of phosphorus pentachloride on an optically active hydroxy acid or its ester is accompanied by a change of sign of rotation, the action of thionyl chloride shows a greater variation, thus:

With Change of Sign.



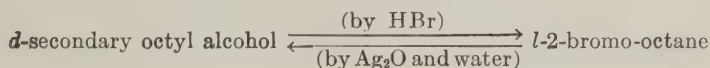
Without Change of Sign.



Two rather remarkable points emerged from a study of the above results. First, the extent of the displacement racemisation which occurs is much greater in those cases when phosphorus pentachloride is the chlorinating agent than when thionyl chloride is used. Secondly, in every instance where the action of thionyl chloride is unaccompanied by a change of sign, there is a phenyl group in direct attachment to the asymmetric carbon atom. A rational explanation of these two regularities is not yet forthcoming.

It was therefore of interest to draw a contrast between the effect produced by an aromatic radical, such as phenyl, and an aliphatic radical, such as methyl, with respect not only to the Walden inversion but also to the displacement racemisation which occurs so very frequently whether accompanied by configurative change or not. From this view-point the changes effected in the mandelic and lactic groups respectively are illuminating in their diversity.⁴

In continuation of our previous work we, therefore, thought it worth while to study the action of thionyl chloride and of thionyl bromide on an optically active aliphatic alcohol, and for this purpose secondary octyl alcohol was selected. The action of hydrogen chloride and of hydrogen bromide on the active alcohol, according to Pickard and Kenyon,^{5,6} is accompanied by a change of sign. We now find, as Levene and Mikeska¹ have also found, that the action of thionyl chloride leads to a change of sign. The same effect is produced by thionyl bromide. Since Pickard and Kenyon have shown that the changes,



can be realised, it seems therefore impossible to bring about the interconversion of the optically active secondary octyl alcohols by the help of thionyl chloride, a reagent which served for the interconversion of the phenylmethyl carbinols.³

EXPERIMENTAL PART.

The secondary octyl alcohols employed were optically pure, and were prepared by the resolution of the *dl*-alcohol by Kenyon's modification⁷ of the original method of Pickard and Kenyon.⁸ The *d*-alcohol had $\alpha_D^{15} = +16.35^\circ$ ($l=2$) in agreement with the value quoted by Pickard and Kenyon ($[d]_4^{17} = 0.8229$, $l=1$, $\alpha_D^{17} = +8.125^\circ$, $[\alpha]_D^{17} = +9.87^\circ$), while the *l*-alcohol which we used had $\alpha_D^{15} = -16.41^\circ$ ($l=2$).

⁴ For a survey of the literature in those groups, compare Walden, P., *Optische Umkehrerscheinungen*, Vieweg, 1919, pp. 48-58.

⁵ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 45.

⁶ Pickard, R. H., and Kenyon, J., *Ber. chem. Ges.*, 1912, xlv, 1592.

⁷ Kenyon, J., *J. Chem. Soc.*, 1922, cxxi, 2540.

⁸ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1907, xei, 2058.

Action of Thionyl Chloride on dl-Secondary Octyl Alcohol.

Kahlbaum's alcohol, which had $\alpha_D = -0.12^\circ$ ($l=2$) and which contained a trace of the levo compound (compare Marckwald and McKenzie⁹), was employed. 12 gm. were added gradually to 20 gm. of thionyl chloride within an interval of 45 minutes, the temperature being kept low. The excess of thionyl chloride was then removed under diminished pressure at the ordinary temperature, and the residue distilled under diminished pressure. The main fraction boiled at $65-73^\circ$ and 21 mm., and contained sulfur. This indicated the probable presence of the sulfinyl chloride and, to decompose this, the oil was heated for 3 hours at 120° when sulfur dioxide was evolved. After distillation, *dl*-secondary octyl chloride was obtained as a colourless oil free from sulfur.

Found. Cl = 24.1. $C_8H_{17}Cl$ requires Cl = 23.9 per cent. b.p. $55-56^\circ$ and 14 mm.

The action was found to proceed more smoothly in the presence of pyridine. The chloride was again obtained free from sulfur.

Found. Cl = 24.1 per cent.

Action of Thionyl Chloride on l-Secondary Octyl Alcohol.

20 gm. of thionyl chloride ($1\frac{1}{3}$ mols) were added within an interval of 35 minutes to a solution of 16 gm. of the *l*-alcohol (1 mol) in 11.2 gm. of pure pyridine. The mixture was kept cold. There was no evolution of gas, and a white crystalline solid separated. After standing at room temperature for 20 minutes, the mixture was heated on the steam bath when a vigorous evolution of sulfur dioxide started. The heating was continued for $3\frac{1}{2}$ hours. Water was added, and the oil then extracted with ether. The ethereal solution was washed with dilute hydrochloric acid, and then with sodium bicarbonate solution, and finally dried with anhydrous sodium sulfate. After fractionation, 9 gm. of *d*-2-chloro-octane were obtained.

Found. Cl = 23.9. Calculated. Cl = 23.9 per cent. b.p. $54-55^\circ$ and 12 mm.

⁹ Marckwald, W., and McKenzie, A., *Ber. chem. Ges.*, 1901, xxxiv, 469.

$$l = 1, [d]_4^{20} = 0.866, \alpha_D^{20} = +29.19^\circ, [\alpha]_D^{20} = +33.7^\circ.$$

This value is considerably higher than that recorded by Pickard and Kenyon⁵ who obtained their chloro-octanes by acting on the active secondary octyl alcohols with hydrogen chloride, in which action the sign of rotation is changed. The values given are $[\alpha]_D^{17} = +20.40^\circ$ and -20.44° respectively. Obviously, the action of hydrogen chloride is attended with considerable racemisation. At the same time, we have no evidence that the above value, $[\alpha]_D^{20} = +33.7^\circ$, is the value for the optically pure compound.

Levene and Mikeska¹ apparently had not carried out the resolution of the *dl*-alcohol quite far enough to obtain the optically pure *l*-alcohol, since the product they used is described as having the low value of $[\alpha]_D = -7.51^\circ$. By the action of thionyl chloride they obtained a dextrorotatory chloro-octane with $[\alpha]_D = +26.68^\circ$ ($l = 1$), but they calculate their specific rotation wrongly when they give it as identical with the observed value.

Action of Thionyl Bromide on d-Secondary Octyl Alcohol.

Thionyl bromide was prepared by passing dry hydrogen bromide into boiling thionyl chloride for 7 hours (Hess and Wahl¹⁰ and Besson¹¹). After purification it boiled at 49° and 30 mm.

29.4 gm. of thionyl bromide (1.4 mols) were added in the course of 45 minutes to a solution of 12.9 gm. of the *d*-alcohol (1 mol) in 11 gm. of pyridine. The subsequent treatment was similar to that adopted in the case of the action of the thionyl chloride, but more prolonged fractionation of the product was necessary. About 4 gm. of *l*-2-bromo-octane were obtained, b.p. $72-73^\circ$ and 18 mm.

Found. Br = 42.3. Calculated. 41.4. This gave $\alpha_D^{16.5} = -5.70^\circ$, ($l = 0.2$).

This product may have been partly racemised owing to the prolonged distillations, since Pickard and Kenyon obtained higher values by the action of hydrogen bromide on the *d*- and *l*-alcohols

¹⁰ Hess, K., and Wahl, O., *Ber. chem. Ges.*, 1922, lv, 2001.

¹¹ Besson, A., *Compt. rend. Acad.*, 1896, cxxii, 320.

respectively. Their values are:

	b. p.	Pres- sure.	$[d]_4^{17}$	$\alpha_D^{17} (l=0.5)$	$[\alpha]_D^{17}$
	°C.	mm.			
<i>d</i> -2-Bromo-octane.....	74	18	1.0895	+14.98°	+27.53°
<i>l</i> -2-Bromo-octane.....	71	14	1.0914	-14.99°	-27.47°

On the other hand, our value of about $[\alpha]_D = -26.1^\circ$ is considerably higher than that of Levene and Mikeska, who by the action of hydrobromic acid on a *l*-alcohol with $[\alpha]_D = -7.51^\circ$ obtained a *d*-2-bromo-octane which they describe as having $[\alpha]_D = +14.56^\circ$.

The action of thionyl bromide on the *d*-alcohol in the presence of pyridine is therefore similar to that of hydrogen bromide inasmuch as a change of sign of rotation results in both cases.

Action of Silver Acetate on d-2-Chloro-Octane.

5.4 gm. of *d*-2-chloro-octane with $[\alpha]_D^{20} = +33.7^\circ$ were heated for 7 hours at 130–140° with 20 gm. of dry silver acetate and 17 cc. of glacial acetic acid. After addition of water, the octyl acetate was extracted with ether and the solution washed with sodium bicarbonate solution, dried with sodium sulfate, and then distilled. After fractionation, 1.5 gm. of a product boiling at 81–82° and 17 mm. were obtained. This was found to be levorotatory, —*l*=0.2, $\alpha_D^{15} = -0.61^\circ$, —but it is partially racemised.

Pickard and Kenyon also found that the action of potassium acetate on the halides caused a change of sign, but the octyl acetates could not be obtained optically pure by this method.

We desire to thank for the assistance rendered the Carnegie Trust for the Universities of Scotland.

THE NITROGENOUS GROUPS OF PLANT NUCLEIC ACID.

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(Received for publication, November 7, 1924.)

Whenever a substance by hydrolysis produces two substances which bear to one another the relation of an oxy-compound to its corresponding amino-compound, the suspicion will arise that the oxy-compound does not really exist as a group in the molecule of the hydrolyzed substance, but is in reality formed by deaminization of the amino-compound or one of its precursors during the hydrolysis. This is especially true where the hydrolytic process employed is a severe one.

The question is a very difficult one to deal with and becomes especially so in certain cases which include the one that we are concerned with in this paper. For to say that an amino-compound formed by hydrolysis will not be deaminized itself by that particular mode of hydrolysis is not to the point; because the amino-compound when once formed may be stable enough under the conditions of the hydrolysis and nevertheless the group of the amino-compound while still in combination may readily undergo deaminization.

Every one will remember that nucleic acid was formerly supposed to contain four purine groups, because the substance under the conditions of hydrolysis employed at that time produced four purine compounds. But it was soon shown that nucleic acid sets free its purine derivatives by very mild hydrolysis when the amino-compounds are produced, but not the corresponding oxy-compounds. Thus the secondary origin of the oxy-purines was proved somewhat easily, but the converse would be almost impossible to prove. That is to say, while the absence of oxy-purine groups from the nucleic acid molecule admits of conclusive proof, the presence of oxy-purine groups by the side of the corresponding

amino-purine groups would be almost impossible to prove with the analytical methods which are at our disposal.

The discussion with reference to the purine derivatives applies equally well to the pyrimidine derivatives. Nucleic acid has been found to yield according to the method of hydrolysis employed

1. Both cytosine and uracil.
2. Both cytosine nucleoside and uracil nucleoside.
3. Both cytosine nucleotide and uracil nucleotide.

But in each of these cases we are dealing with an amino-compound and its corresponding oxy-compound so that what has been stated above in reference to purine groups is applicable here. Does the uracil group actually exist in the molecule of yeast nucleic acid or is the uracil derivative formed from nucleic acid by hydrolysis (in one way or another) to be referred to the cytosine group which alone is present in the nucleic acid molecule?

This question was seriously considered in 1910 by Kowalevsky¹ who concluded that the uracil formed in the severe hydrolysis of nucleic acid is produced not by hydrolysis of the nucleic acid, but by deaminization of the cytosine which is first formed, and therefore the molecule of yeast nucleic acid contains only the amino-pyrimidine group and not the corresponding oxy-pyrimidine group. However indirect and unconvincing the experimental work of Kowalevsky may be considered, her conclusion appears to be correct.

We have attacked the question in an entirely different way. An examination was recently made of the nucleotides that can be obtained from nucleic acid at body temperature by the action of the thermostable ferment of the pancreas. We were somewhat surprised at the comparatively small yield of uracil nucleotide and the correspondingly large yield of cytosine nucleotide obtained.² Now we find that no uracil nucleotide at all is formed, but a large and most unexpected amount of cytosine nucleotide is produced when nucleic acid is decomposed into its nucleotides by the action of dilute sodium hydroxide at room temperature.³ In so far as this result is of value, the conclusion is obvious that the oxy-pyrimidine derivatives (uracil, uracil nucleoside, and uracil

¹ Kowalevsky, K., *Z. physiol. Chem.*, 1910, lxi, 240.

² Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1923, lv, 557.

³ Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1923, lv, 567.

nucleotide) are not referable to an oxy-pyrimidine group in nucleic acid, but are secondary products formed during hydrolysis by deamination of the corresponding cytosine derivatives or their precursors.

EXPERIMENTAL.

Preparation of the Nucleotides from Commercial Nucleic Acid.

50 gm. portions of commercial yeast nucleic acid suspended in 1,250 cc. of warm water and neutralized with sodium hydroxide were treated with such an excess of the reagent as to make the entire alkaline solution 1 per cent, and the product was allowed to digest at room temperature overnight. Determination of ammonia by aspirating the alkaline fluid through a standard acid both before and after the digestion at room temperature showed that the amount of deamination which occurred during digestion was entirely negligible.

After neutralizing the alkaline fluid with acetic acid, the nucleotides were isolated in the usual way through their lead salts, the final filtrate from lead sulfide being evaporated at 40° under diminished pressure to a small volume, hardened with absolute alcohol, and allowed to dry in a desiccator.⁴ Analyses for nitrogen and total phosphorus were made.

I. N = 14.50 per cent.	P = 7.95 per cent.
" = 14.38 " "	" = 7.98 " "
Mean..14.44 " "	Mean...7.97 " "

A second experiment with nucleic acid obtained from a different source gave the following results.

II. N = 14.42 per cent.	P = 7.76 per cent.
" = 14.53 " "	" = 7.85 " "
Mean..14.48 " "	Mean...7.81 " "

⁴ When present with other nucleotides, uracil nucleotide should be precipitated from aqueous solution by the addition of alcohol (Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 111; 1917, xxxi, 39). However, this aqueous-alcoholic solution contains a small amount of a mixture that forms difficultly soluble compounds with brucine. The material may ultimately be found to contain some uracil nucleotide. Its examination is difficult, but its amount is too small to influence the conclusions arrived at in this paper.

Required for:

1 guanine nucleotide group.	} N:P 3.75
1 adenine " "	
1 cytosine " "	
1 uracil " "	

Required for:

1 guanine nucleotide group.	} N:P 4.33
1 adenine " "	
1 cytosine " "	

Required for:

1 guanine nucleotide group.	} N:P 4.00
1 adenine " "	
2 cytosine " "	

Found. I.	4.00
II.	4.10

These results by themselves, of course, do not carry great weight, but they are of considerable significance when taken in connection with what follows.

Separation of the Nucleotides into Two Fractions.

I. The Guanine Fraction.

II. The Adenine Fraction.

The crude nucleotide mixture dissolved in twice its weight of hot water and nearly neutralized with concentrated ammonia was treated with 1.5 parts of absolute alcohol. Under these conditions the nucleotides are separated rather sharply into an adenine fraction and a guanine fraction. The fluid containing the nucleotides of the adenine fraction was filtered with a pump from the guanine cake, treated with lead acetate in slight excess, and the nucleotides were recovered in the ordinary way through their lead salts. Upon evaporation of the filtrate from lead sulfide to a small volume under diminished pressure at 40°, a copious deposition of beautifully crystalline adenine nucleotide occurred. This was filtered off, recrystallized once from hot water, and analyzed with the following results.

I. 0.3472 gm. lost 0.0162 gm. when heated 1 hour at 110–115°.

On exposure to the air, 24 hours, the specimen gained 0.0152 gm.

II. 0.3317 gm. gave 0.2196 gm. $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$.

III. 0.3424 " " 0.2261 " "

IV. 0.1898 " required 10.0 cc. of standard acid (1 cc. = 0.00364 N).

V. 0.1809 " " 9.54 " " " "

	Required for adenine nucleo- tide.	Found.				
		I	II	III	IV	V
H ₂ O.....	4.95	4.66				
P.....	8.49		8.37	8.35		
N.....	19.18				19.17	19.19

The adenine nucleotide was filtered off because its removal makes the subsequent analytical procedure very much easier of execution. Its analysis shows that we did not remove any substance containing uracil nucleotide and also indicates an excellent method of preparing pure adenine nucleotide without the laborious use of its brucine salt. The filtrate from the crystals of adenine nucleotide together with the mother liquor obtained in its recrystallization was allowed to evaporate in a desiccator to a thin syrup, hardened with alcohol, and dried over sulfuric acid. It contained no combined guanine.

Preparation and Fractional Crystallization of the Brucine Salts.

The nucleotide preparation which might be expected to contain both pyrimidine nucleotides and some adenine nucleotide was dissolved in 2 parts of hot water and nearly neutralized to litmus with a hot alcoholic solution of brucine. The usual thick paste of crystalline brucine salts was produced which after cooling was filtered with a pump, washed by grinding to a paste with 50 per cent alcohol, again filtered sharply, and allowed to dry in the air.

The product was then crystallized nine times from hot 35 per cent alcohol. At various stages of the recrystallization nitrogen analyses of the crystallized product were made and the mother liquors were allowed to evaporate in the air for the recovery of the dissolved brucine salts. The results are given in Table I.

This is the crux of the matter. Common experience has shown that when a mixture of brucine salts, containing adenine nucleotide and the two pyrimidine nucleotides, is crystallized repeatedly from 35 per cent alcohol, nearly all of the adenine nucleotide will remain in the first mother liquor. After this crystallization the amount of alcohol required to dissolve the brucine salt of uracil

nucleotide is so large that all cytosine nucleotide originally present will remain dissolved in the cold mother liquors of subsequent recrystallizations, and the final residue after nine recrystallizations will be the pure brucine salt of uracil nucleotide.^{2,5}

But, as in the case here described, where uracil nucleotide is absent and a mixture of the brucine salts of cytosine nucleotide and adenine nucleotide is recrystallized from 35 per cent alcohol, the first mother liquor, as stated, will contain most of the adenine nucleotide which will drag along, and traces will be found in the

TABLE I.

Crystallization No.	Analysis of crystals.	Recovered from mother liquor.
		<i>gm.</i>
1		13.800
2	N = 8.04 N = 8.13	6.600
3		3.300
4	N = 8.10 N = 8.09	3.400
5		3.000
6	N = 7.87	0.500
7	N = 7.89 N = 7.93	0.370
8		0.220
9	N = 7.84 N = 7.88	0.237
Required for:		
Cytosine nucleotide....	N = 7.92	
Uracil "	N = 6.79	

material recovered from subsequent recrystallizations up to the fifth, as can be seen in Table I. The product obtained after six recrystallizations is evidently the pure brucine salt of cytosine nucleotide and does not change its composition however much it is afterwards recrystallized.

Recovery of the Nucleotides from Their Brucine Salts.

The nucleotide was prepared from the brucine salt obtained by evaporation of the first mother liquor (13.8 gm.). As was to be

⁵ Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425; 1919, xl, 415.

expected, a copious yield of beautifully crystalline adenine nucleotide was obtained.

The brucine salts obtained by evaporation of the mother liquors from the second, third, fourth, and fifth recrystallizations were rejected.

The brucine salt obtained from the mother liquors of the sixth, seventh, eighth, and ninth recrystallizations joined to the final residue was used for the preparation of the free nucleotide in the ordinary way through its lead salt. Cytosine nucleotide was finally obtained in beautiful transparent plates which on hydrolysis did not produce a trace of any purine compound precipitable with silver nitrate and ammonia, and lost no weight when heated at 110°. After recrystallization once from hot water the substance was analyzed.

0.2653 gm. required 9.55 cc. of standard acid (1 cc. = 0.003637 N).

0.2256 " " 8.13 " " " "

0.2040 " " 7.36 " " " "

	Required for cytosine nucleotide.	Found.		
		I	II	III
N.....	13.00	13.09	13.10	13.13

It is interesting to recall that in a recent investigation of the nucleotides isolated from the pancreas by the method here described (that is, by the action of dilute sodium hydroxide in the cold), guanine nucleotide, adenine nucleotide, and cytosine nucleotide were shown present, but uracil nucleotide could not be found. There is, of course, no assurance that the plant nucleotides of the pancreas are present in combined form as plant nucleic acid, but taken in connection with the present paper the result is significant.

SUMMARY.

When yeast nucleic acid is treated with dilute sodium hydroxide at room temperature it is decomposed into its nucleotides without the formation of any free phosphoric acid or free purine bases, and deamination does not occur.

Among the products, three of the hitherto accredited nucleotides of yeast nucleic acid (guanine nucleotide, adenine nucleotide, and cytosine nucleotide) can easily be isolated in quantity, but the fourth nucleotide (uracil nucleotide) cannot be found. This is especially remarkable because, of the four nucleotides, uracil nucleotide is the one least likely to be lost in analytical procedures, on account of the great insolubility of its brucine salt in 35 per cent alcohol.

The conclusion seems obvious, that the uracil derivatives which have hitherto been obtained from yeast nucleic acid, are secondary to the corresponding cytosine derivatives.

THE EFFECT OF SODIUM BENZOATE INGESTION UPON
THE COMPOSITION OF THE BLOOD AND URINE WITH
ESPECIAL REFERENCE TO THE POSSIBLE SYNTHESIS
OF GLYCINE IN THE BODY.

PRELIMINARY PAPER.

By W. W. SWANSON.

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of Minnesota, Minneapolis.)

(Received for publication, June 26, 1924.)

Since the work of Wiechowski (1) in 1906 several investigators have confirmed the fact that the excretion of urea is decreased after ingestion of sodium benzoate. In 1916 Lewis and Karr (2) found that uric acid was also markedly decreased in the urine under similar conditions. From these findings it was suggested that, where there is an abnormal demand for glycine, this amino acid is synthesized, in part, from the end-products of protein metabolism.

Recent experiments by Gibson and Doisy (3) indicate that some organic acids such as lactic and benzoic have the tendency to decrease the excretion of uric acid and to cause a slight increase of the same in the blood. However, figures showing the effect of the ingestion of sodium benzoate were not included in their paper.

It is the aim in this paper to present values for the non-protein nitrogen constituents in the blood and urine obtained by simultaneous analyses after ingestion of sodium benzoate; further, to investigate what bearing these values may have, if any, on the synthetic ability of the human body to produce glycine for conjugation with the benzoic acid.

Analytical Methods.

All the experiments were done on young healthy individuals. The blood was drawn from an arm vein and immediately transferred to a test-tube containing 20 mg. of potassium oxalate per

10 cc. of blood to prevent clotting. Filtrates were always prepared a few hours after being drawn and in every instance analysis of the blood was completed during the ensuing 24 hours. Drawn blood as well as filtrates were always kept in the refrigerator to minimize changes in composition. The preparation of protein-free blood filtrates and determination of total non-protein nitrogen, urea, and sugar were done according to directions found in Folin's Manual (4). Duplicate analyses were always made and, if these did not check, a second determination was completed. The uric acid in the blood was determined by the new method of Benedict (5). The amino acid nitrogen determination was carried out on the same filtrates using Folin's (6) colorimetric method, and the plasma proteins by the colorimetric method of Wu (7).

In order that the data presented should be more complete, hourly specimens of urine were taken during the experimental period. A 4 hour morning specimen was generally used as control. The urines were preserved by adding 2 cc. of a 2 per cent nitric acid solution and a few drops of a 10 per cent solution of thymol-chloroform.

The amino acid nitrogen determination in urine was made as described by Folin (8) on hourly specimens. The determination of hippuric acid was carried out by the method of Kingsbury and the author (9), uric acid by the method of Benedict and Franke (10), and urea nitrogen by the method described in Folin's Manual (4).

To insure uniformity and obviate the influence of food, a slice of toast and a cup of coffee were taken in the morning, but no further food during the time of the experiment. There was no restriction placed upon the water intake, and in none of the experiments did the intake exceed the ordinary amount for the individual.

EXPERIMENTAL.

Tables I and II are submitted as controls for the work to follow. The constituents listed for blood and urine are well within the normal range when compared with values reported in the literature.

In Tables III, IV, and V are shown the values for blood and urine after ingestion of 3 to 4 gm. of sodium benzoate. Some

TABLE I.*

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.					Plasma.				
	Amino acid N.	Urea N.	Total non-protein N.	Uric acid.	Sugar.	Amino acid N.	Urea N.	Total non-protein N.	Uric acid.	Sugar.
<i>p.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
12.20	8.3	17.0	37	3.0	90	6.4	16.6	23	3.6	97
6.15	7.0	16.6	33	3.0	83	6.0	16.6	22	3.4	91

* Average of a series of five experiments.

TABLE II.*

Subject, W. W. S. Weight, 76 kilos.

Analysis on 1 hr. specimens of urine.

Time.	Volume per hr.	Amino acid N.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>
6.40 to 11.30	54	4.2
<i>p.m.</i>		
12.30	58	4.7
1.40	60	3.7
3.30	38	3.2
4.30	36	3.2

* Average of a series of three experiments.

TABLE III.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.			Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Urea N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
<i>a.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>cc.</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>
7 20 to 11.00*	6.6	19	33	5.5	16.7	30	46	6.7	0.084	0.035
<i>p.m.</i>										
12.00	6.4	14	30	5.5	15.0	27	71	5.9	0.994	0.44
1.00	6.4	15	32	5.9	13.0	28	253	5.1	1.85	0.77
2.00	5.7	15	30	5.9	14.0	30	77	3.9	0.73	0.31
4.30	5.7	15	30	6.2	15.0	27	46	3.5	0.18	0.07

* Took 3.34 gm. of sodium benzoate.

TABLE IV.

Subject, H. H. J. Weight, 86 kilos.

Time.	Whole blood.			Plasma.		Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
<i>a.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>cc.</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>
11.30*	9.0	13.0	30	7.3	35	60	6.0		
<i>p.m.</i>									
12.30	7.9	14.4	41	7.0	34	140	5.4	1.56	0.652
1.30	8.5	11.4	41	7.6	35	405	6.3	1.89	0.794
2.30						88	4.5	0.62	0.262
3.30	8.2	13.0	40	8.4		50	5.0		
5.00						26	3.8		

* Took 3.65 gm. of sodium benzoate.

TABLE V.

Subject, B. B. Weight, 76 kilos.

Time.	Whole blood.			Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
<i>a.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>cc.</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>
6.40 to 11.15*	11.0	12.5	36	86	8.2		
<i>p.m.</i>							
12.15	8.3	15.7	34	106	6.3	1.31	0.553
1.15				95	4.8	1.57	0.658
1.20	8.7	12.0	31				
3.15	8.7	16.0	32	41	3.4	1.05	0.440

* Took 3.5 gm. of sodium benzoate.

TABLE VI.

Subject, A. G. M. Weight, 65 kilos.

Time.	Whole blood.			Plasma proteins.		
	Amino acid N.	Urea N.	Total non-protein N.	Fibrin.	Globulin.	Albumin.
<i>p.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
12.20*	8.2	16.6	48.4	0.22	2.1	5.2
2.10	7.4	10.8	31.2	0.23	2.0	5.1
3.30	9.3	12.5	43.2	0.22	2.0	5.2

* Took 3.30 gm. of sodium benzoate.

variability appears to exist in the values obtained for the different individuals. These variations are, however, too small to draw any definite conclusions from, but may indicate to some extent the effect of the benzoate upon normal values in blood and urine.

TABLE VII.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.			Plasma proteins.		
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Urea N.	Total non-protein N.	Fibrin.	Globulin.	Albumin.
<i>p.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
12.00*	7.2	23.0	46	6.4	19.4	28.4	0.32	2.2	4.7
2.00*	7.0	17.5	42	4.5	18.7	26.0	0.33	2.3	4.4
4.00	5.9	16.5	34	4.9	18.0	25.0	0.33	2.3	4.4
6.00	5.5	17.6	34	5.0	20.0	25.0	0.34	2.3	4.7

Urine per hr.

Time.	Volume per hr.	Amino acid N.	Urea N.	Uric acid.	Hippuric acid.	Glycine.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>
7.00 to 11.50	60	7.5	540	62		
<i>p.m.</i>						
12.00*						
12.50	198	7.2	166	13	1.03	0.43
1.50	215	5.5	150	11	1.25	0.52
2.00*						
2.50	170	4.9	136	8	1.44	0.60
3.50	172	5.3	136	7	1.56	0.65
5.00	134	4.6	134	9	2.86	1.20
6.30	90	4.3	135	15	1.13	0.47

* At 12.00 n. took 3.35 gm. and at 2.00 p.m., 6.70 gm. of sodium benzoate.

In Tables VI and VII is shown the stability of plasma proteins under varying quantities of ingested sodium benzoate. The blood values in Table VII with a trebled dose of sodium benzoate differ in no particular from those listed in Tables III, IV, and V.

A more complete analysis of the urine in Table VII confirms again the established facts that urea and uric acid are markedly decreased after ingestion of benzoate.

TABLE VIII.

Subject, A. G. M. Weight, 65 kilos.

Time.	Whole blood.				Plasma.			
	Urea N.	Uric acid.	Total non-protein N.	Sugar.	Urea N.	Uric acid.	Total non-protein N.	Sugar.
<i>p.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
12.40*	19	4.2	34	98	19	4.8	26	130
2.40	19	4.8	33	90	20	5.3	23	118
4.40	19	4.8	42	83	20	5.5	29	100

Urine per hr.

Time.	Volume per hr.	Urea N.	Uric acid.
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>
8.00 a.m. to 12.40 p.m.*	40	420	28
<i>p.m.</i>			
1.40	51	216	9
2.40	65	140	8
3.40	163	195	9
4.55	40	150	12

* Took 5 gm. of sodium benzoate.

TABLE IX.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.		
	Urea N.	Uric acid.	Total non-protein N.	Urea N.	Uric acid.	Total non-protein N.
<i>p.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
12.40*	19.0	3.6	33.0	17.0	4.2	25
2.40	15.4	4.0	31.0	16.8	4.5	22
4.40	15.0	4.0	36.0	16.0	4.5	22
5.40	14.0	4.4	30.0			

Urine per hr.

Time.	Volume per hr.	Urea N.	Uric acid.
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>
7.30 a.m., to 12.40 p.m.*	35	580	24
<i>p.m.</i>			
1.45	90	332	6
2.50	85	280	4
3.50	200	380	7
4.50	345	345	9
5.50	66	228	14

* Took 10 gm. of sodium benzoate.

To show just what happens to the blood uric acid Tables VIII, IX, X, and XI are submitted. There is a definite rise in the uric acid content of both the whole blood and plasma, while the urine analyzed simultaneously shows the marked decrease.

TABLE X.

Subject, Wm. K. Weight, 70 kilos.

Time.	Uric acid.		Urine per hr.	
	Whole blood.	Plasma.	Volume per hr.	Uric acid.
<i>a.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>cc.</i>	<i>mg.</i>
7.15 to 8.15			22	18
8.00*	4.3	5.3		
8.35	4.3	5.3		
10.45			52	3
<i>p.m.</i>				
1.30	4.3	5.3	52	3
3.30	4.7	5.7	37	4

* Took 10 gm. of sodium benzoate.

TABLE XI.

Subject, Wm. K. Weight, 70 kilos.

Time.	Uric acid.		Urine per hr.	
	Whole blood.	Plasma.	Volume per hr.	Uric acid.
<i>a.m.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>cc.</i>	<i>mg.</i>
7.00 to 8.00			24	18.0
8.30*	4.0	5.0		
10.30			60	2.7
12.00	4.2	5.3	52	2.0
<i>p.m.</i>				
2.30	4.7	5.2	33	2.5

* Took 10 gm. of sodium benzoate.

DISCUSSION.

From the experimental findings there may be some support for the supposition that the constituents, which during ordinary metabolism enter into the composition of urea, may in part be synthesized into glycine. If it were merely a retardation of excretion, then there should be an increase of urea nitrogen in the blood. This, however, is not the condition, for in most experiments the blood urea nitrogen is slightly decreased.

The values obtained for uric acid in blood lead to a different conclusion. While the experiments are few in number, there appears to be a gradual increase in uric acid in the blood and plasma after benzoate ingestion. This fact seems to contradict the suggestion of Lewis and Karr (2) who thought that a decrease of the uric acid in the urine might mean a possible hydrolysis of this acid and synthesis of the resulting products into glycine. It is more probable that the excretion of uric acid is slightly retarded whenever large amounts of hippuric acid are being eliminated.

It was hoped that a study of the blood and urine amino acid content, after sodium benzoate ingestion might throw some light on the nature of the circulating amino acids; that is, if the total amount of these was moderately lessened at the time of rapid hippuric acid formation, it would be reasonable to believe that the circulating amino acids were largely composed of glycine. Experiments recorded in Tables III, IV, and VII, showing little or no appreciable difference in the plasma amino acid after relatively large doses of sodium benzoate, indicate that this is not the case. The urine amino acid per hour shows a decrease after benzoate ingestion in all the experiments; but this is also noted in the experiment shown in Table II in which no benzoate had been taken and is due merely to inanition.

SUMMARY AND CONCLUSION.

After ingestion of sodium benzoate the simultaneous analyses of blood and urine of normal individuals show: (1) no increase in the urea nitrogen content of the whole blood and plasma, but a decrease in the urea nitrogen of the urine; (2) an increase of the uric acid content of the whole blood and plasma, and a marked decrease in the uric acid content of the urine; (3) the glycine portion of the blood amino acids and of those which are normally excreted while fasting appears to be only a small percentage, if any, of the total; and (4) the experimental findings suggest a probable synthesis of glycine from the constituents which normally are converted into urea, but it appears improbable that uric acid supplies any glycine.

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FAT-SOLUBLE VITAMINS.

XXI. OBSERVATIONS BEARING ON THE ALLEGED INDUCTION OF GROWTH-PROMOTING PROPERTIES IN AIR BY IRRADIATION WITH ULTRA-VIOLET LIGHT.*

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In 1922 Hume (1) and Goldblatt and Soames (2) pointed out that irradiation of experimental animals on a diet free from fat-soluble vitamins led to stimulation of growth. On the basis of their observation Steenbock and Nelson (3) laid the foundations for a method which allowed vitamin A and, under certain conditions, the antirachitic vitamin to be determined—using growth in rats as the criterion. They showed that the antirachitic vitamin as well as vitamin A is required for growth under the conditions which they and the English investigators conducted their experiments. In this method the rats are put upon a basal ration free from fat-soluble vitamins except possibly for the material to be analyzed for these vitamins, and then the rats are periodically exposed to ultra-violet light to furnish them with the required amounts of the antirachitic factor. When growth ceases under these conditions it is assumed to be due to exhaustion of the animal's reserve, as well as of the ration's supplement of vitamin A. This method has been used satisfactorily in this laboratory in a large number of experiments.

While the above experiments were in progress, Hume and Smith (4) published a paper again bringing to the fore the alleged physiological activity of air which had been exposed to ultra-violet

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

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light. They concluded that air thus exposed was growth-stimulating to rats when fed a diet deficient in fat-soluble vitamins.

The experimental procedure described by Hume and Smith led the writers to infer that the conclusions arrived at were open to question. They were strengthened in their convictions by results obtained by Jones, Steenbock, and Nelson¹ when attempting to use the aforementioned method of the writers for the determination of vitamin A and the antirachitic vitamin in cod liver oil and butter fat. As already related¹ in those experiments, irradiated and non-irradiated animals grew approximately equally well, contrary to results obtained in dozens of other experiments. Inasmuch as in these experiments irradiated and non-irradiated animals had been kept in the same cage, which procedure had not been adopted before, it suggested itself that the irradiated animals had by their presence had some effect upon the growth of the non-irradiated controls. Accordingly, various suggestions carried by the literature from other fields of endeavor were entertained.

It is always a dangerous matter to attempt to interpret another investigator's findings, because in no case are all the conditions possibly bearing on the outcome of an experiment ever recorded, but, nevertheless, it suggested itself to the authors that Hume and Smith (4) had overlooked the possible rôle of the sawdust with which their animals had been bedded. It is true that they report negative experiments where the irradiated air was blown out of the jars, in which the animals were kept, with a bellows, but unfortunately no mention is made of whether or not the sawdust was thereby removed. Furthermore, no mention is made by the English writers whether or not food and water were removed from the jars previous to irradiation. In view of results, published and unpublished, from this laboratory, this would be of import in evaluating their results (5).

A search of the literature revealed the fact that zinc plates and paper have been made photochemically active by exposure to sunlight to the extent that they conveyed an image to a photographic plate (6). It was not an impossibility that these phenomena, observed by us, might be a similar case of secondary radiation, as has already been expressed in a publication from this laboratory in connection with the action of the antirachitic vitamin,

¹ Jones, J. H., Steenbock, H., and Nelson, M. T., unpublished data.

and which theory is apparently supported by recently published data of Kugelmass and McQuarrie (7).

With this in view the authors proceeded in initial experiments essentially according to the technique of Hume and Smith (4). However, instead of sawdust the rats were kept on screens according to the technique used for vitamin B determination in this laboratory (8) and particular attention was given to removal of food, screens, and excreta from the jars previous to irradiation. In the first experiment six rats from one litter were subjected to the following treatments: two rats were directly irradiated; two were put on an irradiated screen; and two on a non-irradiated screen. In the first case the rats were irradiated in a jar on the screen. The air was not blown out. In the second case the screen was irradiated in the jar. In the third case the screen was put in after the air in the jar had been irradiated. In all cases the jars were cleaned beforehand and food and water were removed. The air was also blown out of the jars after the irradiation except where the rats were directly irradiated.

The results of these experiments were that just as good growth was obtained when the rats were placed upon the irradiated screen, even though the irradiated air had been blown out, as when they were directly irradiated. When put upon the non-irradiated screen, no growth resulted. In our next experiments we obtained direct evidence that irradiated air in the absence of an irradiated screen or irradiated food had no effect. While this to us at that time pointed unequivocally to an activation of the screen, our later experiments, as detailed in the experimental part, have forced us to accept another point of view.

EXPERIMENTAL.

The experiments here reported depended for the most part upon growth as an indication of positive or negative effect. Inasmuch as under the experimental conditions imposed the authors have seen fit to associate lack of growth with an absence of the anti-rachitic factor (3), analysis of bones for ash were made on all animals with the termination of the experiments. Finally, histological observations were also made on the effect of various factors on the deposition of lime salts in rachitic bone, thus relating the physiological to the pathological.

In all the growth experiments young white rats, 21 to 25 days old and weighing from 40 to 55 gm., were used as the experimental animals. They had been reared on a special ration (9) under conditions designed to impoverish them in their stores of the antirachitic vitamin without depletion of reserves of vitamin A. In all cases controls were run on other rats from the same litter and only such litters were selected in which the sexes could be uniformly distributed among the different groups. The experiments were usually terminated at 5 weeks, because at that time the animal's stores of vitamin A were exhausted and growth was being inhibited. The exhaustion of these reserves was indicated by the incidence of ophthalmia and infections of the respiratory tract.

In our growth experiments the rats were fed a ration composed of alcohol-extracted and heated casein 18, agar 2, yeast 6, salt 40 (3), 4, and dextrin 70. When fed on this ration the rats usually exhausted their reserves of antirachitic vitamin in 2 weeks as indicated by cessation of growth.

Light from a quartz mercury vapor lamp was used for the irradiation as already described (3). When the animals were directly irradiated they were exposed 10 minutes at a time, at first daily, and later 6 days out of the week. In all cases irradiation was carried out at a distance of about 2 feet.

Effect of Irradiated Rats upon the Non-Irradiated.—While the authors were convinced of the correctness of the conclusions of Jones, Steenbock, and Nelson,¹ it appeared desirable to have available a most carefully controlled demonstration of the fact that irradiated rats, kept in the same cage with non-irradiated rats, are able to stimulate growth in the latter. For this purpose non-irradiated rats were kept alone and with irradiated rats from the same litter with the results shown in Chart I. It leaves no doubt as to their stimulating effect upon the non-irradiated animals.

Effect of Irradiation of Galvanized Iron Screens.—These experiments represented the first attempts to show that possibly the sawdust used as bedding for the animals was responsible in Hume and Smith's experiments (4) for such positive results as they obtained. Irradiation of the screen on which the animals were kept even in the absence of irradiated air was found by us to be responsible for our observed stimulation of growth. In these

experiments and in the following, with two exceptions as noted in the text, the rats were kept in large battery jars 9 inches in diameter and 14 inches high, two rats in each jar. These jars were equipped with galvanized iron screen bottoms of No. 19 wire, 3 meshes to the inch, which were raised from the bottom of the jar about 1 inch by their overturned edges. Food was provided in a tin cup and water from a glass drinking fountain. Each jar was irradiated 10 minutes every 2nd day after being washed and dried. The first jar contained the rats and screen during the

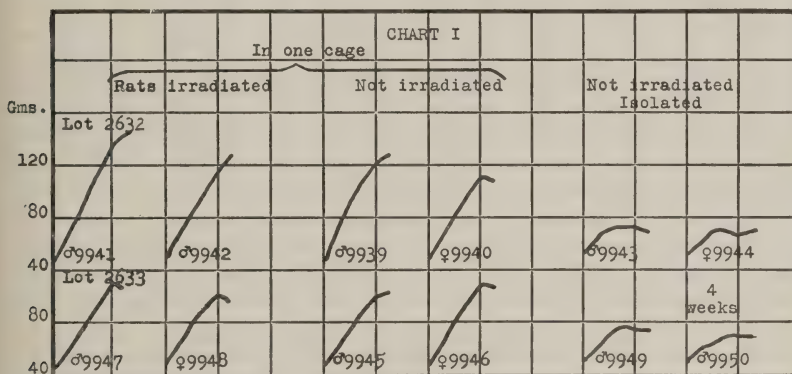


CHART I. Rats exposed to ultra-violet light for 10 minutes every other day grew no better than non-irradiated animals kept in the same cage, but attained a considerably greater weight than non-irradiated rats from the same litter kept separately. Rats 9941, 9942, 9939, and 9940 were kept in one cage; Rats 9947, 9948, 9945, and 9946 in another; and Rats 9943, 9944, 9949, and 9950 in a third. These cages, 2 feet square and 20 inches high, were provided with false screen bottoms, 3 meshes to the inch, which, as we believed, did not permit access of non-irradiated animals to the excreta of the others.

irradiation; the second the screen only; and the third was empty. The air was blown out of the second and third jars after each irradiation after removal to another room. Chart II brings out the fact that the irradiated screen in the absence of irradiated air had the same effect as direct irradiation of animals.

Irradiation of Copper Screens.—When it had been demonstrated that an irradiated galvanized iron wire screen exerted a growth-stimulating influence, the question arose as to whether this property could be induced only with this wire or whether it could be

induced with some other metal as well. Chart III shows that copper could be activated fully as well as zinc. The same precautions were observed in this experiment as in the preceding one in regard to removal of food, water, irradiated air, and in regard to cleanliness of jars.

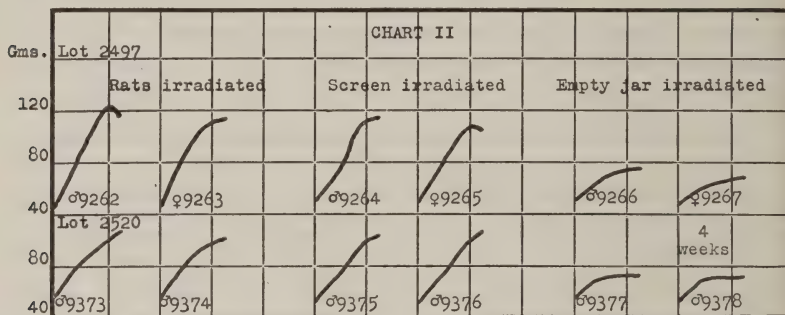


CHART II. Irradiating a galvanized iron screen bottom in a glass jar in which the rats were kept had the same growth-promoting influence as direct irradiation of the rats. The jar when irradiated contained nothing but the screen and any irradiated air was removed by holding the jar before a 10 inch electric fan for 1 minute. When the empty jar was irradiated and the air blown out there was little or no growth after the 2nd week. Lot 2520 was started on this experiment after Lot 2497 was discontinued and bears out the previous results with uniformity.

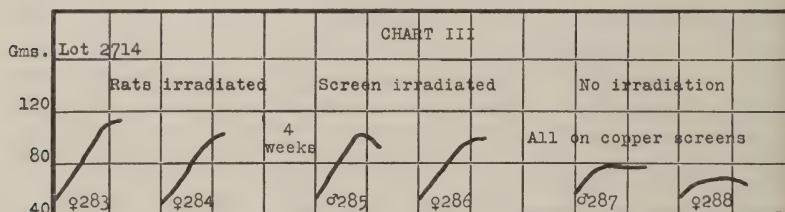


CHART III. The use of a copper screen in place of galvanized iron did not change the results. The growth of Rats 285 and 286 was nearly on a par with the rats irradiated directly and was decidedly better than the growth of the non-irradiated rats. "No irradiation" in Charts III, IV, and VI refers to rats only. As a matter of fact, the empty jar was irradiated (see Table II, Lots 2714, 2532, and 2642).

Is the Induced Activation an Electrical Effect?—It is a well known fact that when certain metals are exposed to ultra-violet light, they give off electrons and take on a positive charge. Since we were entirely in ignorance of what factors might be involved in

activating the irradiated metal, the possibility of an electrical effect suggested itself. The results of experiments bearing on this are presented graphically on Charts IV and V. The former represents a repetition of Experiment II with the modification that the screen was connected to a water pipe to ground it during the irradiation. In the experiments of Chart V galvanized iron

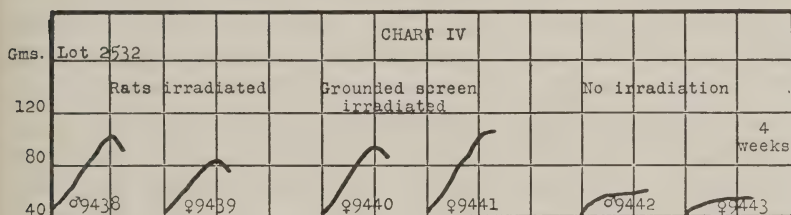


CHART IV. Grounding the screen, by connecting it to a water pipe with a copper wire to prevent the accumulation of an electric charge during irradiation, did not prevent it from acquiring the growth-promoting property. The differences in growth in the first four animals in this chart are normal variations which may be expected in a litter and need no particular comment.

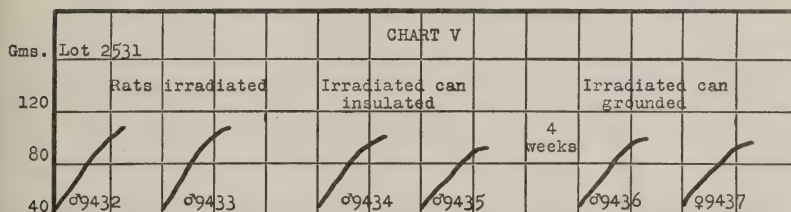


CHART V. A verification of the results in Chart IV is shown in this chart. These animals were kept in galvanized iron cans of the same size and shape as the glass jars used in previous experiments. The first two rats represented were irradiated directly, the next two were kept in a can which was kept insulated, and the remaining two rats were kept in a can which was grounded during the irradiation. The insulated can was kept in a glass dish during and after irradiation. Nothing was permitted to touch it which would remove a charge until it was washed, previous to another irradiation. The rats were dropped into the can, while food and water additions were made with rubber-covered forceps. The uniformity of growth of this litter is exceptional and shows definitely that the acquisition of the growth-promoting quality of the galvanized iron during irradiation is not due to an accumulation of an electrical charge during exposure to light.

cans were used in place of glass jars for the containers of the animals. The charts show that the prevention of the accumulation of an electrical charge, or conversely, the prevention of a discharge, did not influence the nature of the results obtained.

Effect of Irradiated Air.—As the work progressed, evidence accumulating tended to show that Hume and Smith (4) might very well have been in error in regard to their conclusions as to the effect of irradiated air. Their technique is not described fully enough to permit us to interpret their experiments with finality. In a direct attempt to confirm their findings we were unable to demonstrate that irradiated air had any growth-promoting properties (Chart VI). In this experiment two rats were kept in a covered jar containing air irradiated every other day

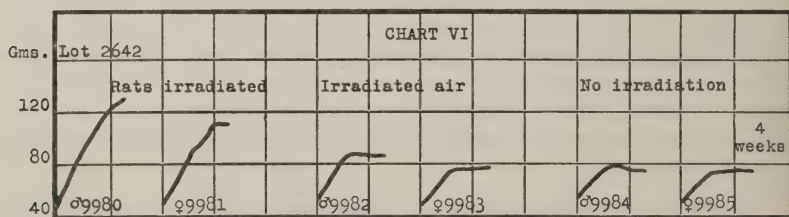


CHART VI. Irradiated air had no effect on the growth of rats on a diet free from fat-soluble vitamins. Rat 9982 grew a little more than the other non-irradiated rats, but this is of no significance. Without exposure to ultra-violet light growth ceased in 2 weeks. See also in this connection Charts XI and XII where the air factor was disregarded.

for 10 minutes and then the cover was removed. These animals grew no better than rats kept in a jar from which the irradiated air had been immediately removed with a fan as previously described. By way of contrast the rats irradiated directly never failed to grow.

Effect of Irradiated Granulated Zinc in Quartz and Jena Flasks.—An attempt to demonstrate the activity of irradiated granulated zinc kept in quartz or Jena flasks was unsuccessful. Two litters of six rats each were divided into six pairs with one rat from each litter. Each pair was kept in a glass battery jar 6 inches in diameter and 8 inches high. Each jar was provided with a false galvanized iron wire screen bottom. The quartz or Jena flasks were suspended from the screen covers with which these

jars were provided. Maximum effect, if any, was to be expected, because, due to the small size of the jars in which they were confined, the rats could not get farther away from these flasks than approximately 2 inches and most of the time they were in actual contact with them. In one case the zinc was irradiated in an open dish before it was put into the flasks to make certain that the metal was adequately exposed and the possible activation was

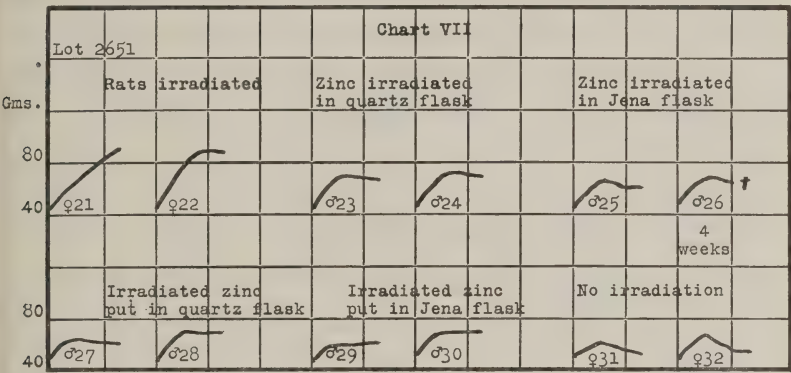


CHART VII. Irradiating a quartz flask containing 50 gm. of granulated zinc and keeping it in immediate contact with the rats did not enhance their growth. No better results were obtained by removing the zinc and making the exposure to light direct. Substituting Jena flasks for quartz made no difference. The zinc used was J. T. Bakers' Special zinc metal, containing no arsenic or cadmium and 0.005 per cent each of lead and iron. Small jars were used to reduce the distance of the rats from the exposed zinc. Two litters of four males and two females each were used and one rat from each litter put in a jar. It is to be noted that the controls, both irradiated and non-irradiated, are females. Due allowance must be made for this in comparing the curves of growth. Rat 26 died before the termination of the experiment, as indicated by a cross at the end of the growth curve.

not hindered by any impermeable medium. The results of these experiments shown in Chart VII are all negative and demonstrate that the growth-promoting radiations of the zinc (if at all existent) were not able to penetrate the glass or quartz, or most certainly, that some other explanation must be formulated.

Effect of Wire Screen Irradiated in Quartz and Jena Flasks.—Inasmuch as the previous experiment dealt with the activation of pure zinc in quartz and glass, it suggested itself that the experi-

mental procedure could be improved upon by using galvanized iron wire screen instead (such as used in the cages)—the other conditions being kept the same.

It was also recognized that there must be entertained the possibility that a used screen might carry the activation which could not be carried by an unused screen. To meet these possibilities one flask in each case was filled with galvanized iron wire screening which had already been used in a rat cage and had not been cleaned. In the main the technique was the same as in the pre-

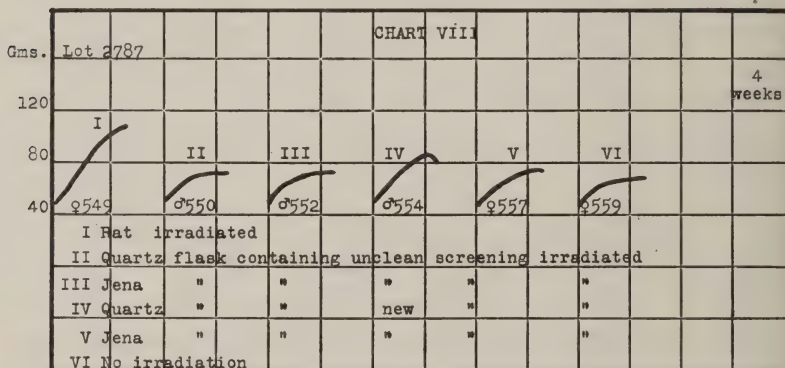


CHART VIII. Galvanized iron screening, both new and soiled, taken from a screen previously used as a false bottom in other cages, when irradiated did not promote growth. 50 gm. of screening was cut up with tin shears and put into 300 cc. flasks, Jena or quartz, and then suspended in small battery jars 6 inches in diameter and 8 inches high. Each jar contained one rat. Only the control rat, directly irradiated, grew. The rats exposed to the irradiated screen did not grow any better than the non-irradiated control.

ceding experiment. All irradiations were conducted with the screen in the flasks. Chart VIII shows that such screening was as inactive as the new.

Effect of Providing a New Screen before Each Irradiation.—It was very evident from the negative results obtained, as reported above, that contact of the animal with the screen or access thereto was a matter which merited consideration in the next experiments, and in this connection the matter of contamination of the screen by the animal which could be activated was given prominence. To this end rather than attempt to clean the screens, a new screen

was taken for every irradiation. Chart IX shows that under these conditions negative results were obtained, suggesting that it was not the screen itself but such contamination of the screen as was caused by the animal that was responsible for the effect.

Effect of Irradiated Screen Placed above the Animals.—It is to be admitted that we were surprised at the suggested importance of the small amounts of foreign material contaminating the screen in our experiments. We were entirely cognizant of this contamina-

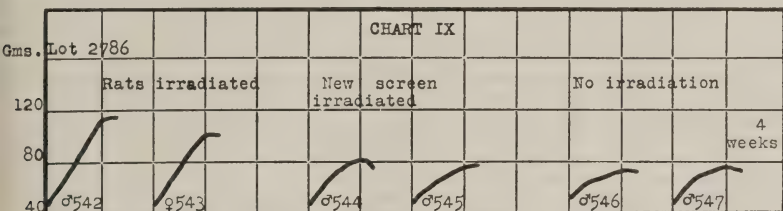


CHART IX. The use of a new screen before each irradiation caused the complete absence of the growth-promoting effect previously observed.

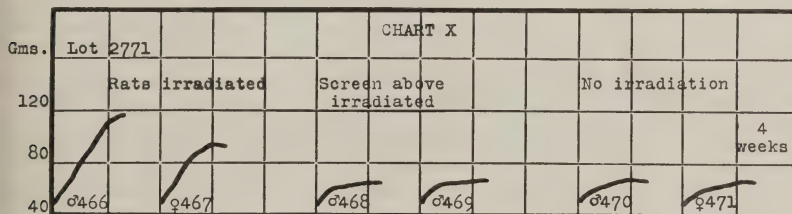


CHART X. A screen previously used in the bottom of a jar and not cleaned had no effect when irradiated and placed 6 inches above the screen bottom in the jar. The rats were prevented from coming in contact with the irradiated screen by a supporting screen placed below. This was also made of the same material and same size mesh.

tion, for while casual inspection did not impress us with their uncleanness, it was noticed that they acquired an unclean feel to the touch. They were, however, not cleaned oftener than once a week when they were scrubbed with a brush in hot water.

In the present experiments an attempt was made to determine if actual contact of the animals with the irradiated screen was necessary to secure the stimulation of growth. To this end an unclean screen which had previously served as a false bottom for a cage was irradiated 10 minutes every other day and sus-

pended above a group of animals at a distance of 6 inches. Contact of the animals with this screen was prevented by the insertion of an intervening screen partition. Chart X shows that a screen thus placed was devoid of activity even though soiled by the animal and irradiated.

Effect of an Irradiated Washed Screen and Miscellaneous Observations.—In Chart XI are shown the results of miscellaneous trials carried out on two litters of rats with the irradiation intensified to 10 minutes daily 6 days of the week—using two rats as controls without irradiation. Two rats were irradiated directly to

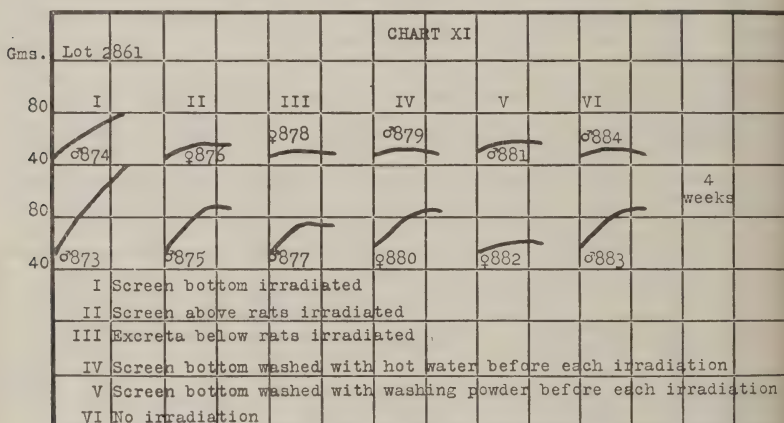


CHART XI. Attempts to confer growth-promoting properties to substances not within reach of the animals were unsuccessful. When the screens upon which the animals were kept were washed before irradiation there was no growth promotion. Six jars as numbered on the chart were used in this experiment. Two litters of rats were used. On the chart, growth curves of animals in the same jar are placed in vertical alignment and those in the same litters in horizontal alignment. All irradiations were 10 minutes daily. The screen bottom in Jar I was irradiated directly as in the experiments shown in Chart II. Jar II contained an irradiated screen above the animals as in Chart X. Jar III contained an 8 inch watch-glass irradiated without cleaning and placed below the bottom screen. The screen in Jar IV was washed with hot water before irradiation and the screen in Jar V washed with hot water and Gold Dust. Jar VI contained the non-irradiated controls.

In Jars IV and V the irradiated air was not blown out and the fact that the rats did not grow offers additional evidence that irradiated air is inactive.

bring out the maximum response possible. The others, two by two, were subjected respectively: (a) to the presence of an irradiated screen overhead; (b) to the presence of irradiated excreta underneath; (c) to an irradiated screen bottom washed with hot water; and (d) to an irradiated screen bottom washed with an alkaline washing powder (Gold Dust). As the chart indicates all trials except the irradiated control gave negative results.

Chart XII emphasizes, in the pronounced difference in growth in the various animals, the inactivity of screens irradiated after

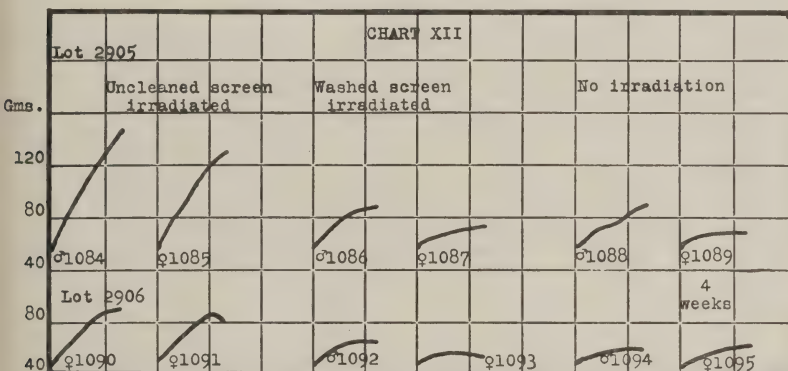


CHART XII. Thorough washing of the screens with hot water and Gold Dust before irradiation prevented them from conferring upon the animals the growth-promoting effect. The screen upon which the first two animals in each lot were kept was irradiated 10 minutes daily without washing. The next two animals in each litter were kept on screens which had been washed before each irradiation, and the remaining animals were non-irradiated controls. Lot 2905 grew considerably more than Lot 2906, but the differences within each litter are of about the same order. As in Chart XI no precaution was taken to remove irradiated air.

washing. It might be said that failure to recognize in our earlier experiments the possible factor of screen contamination was a serious technical mistake, because in all other respects the utmost precautions had been taken. In view of what we now know this is to be admitted, but at the time it was not evident to us. As a matter of fact the contamination of the screens was of so slight an order that a casual observer comparing the washed screens with the unwashed would not have noticed the difference. All the animals that we used were, of course, small animals and

the solid excreta were voided in very small pellets so that contamination of the screen with them was a rare occurrence. We do not as yet know the mechanism whereby the animal is directly affected, but we believe that the animal is directly stimulated by the consumption of small amounts of the activated contamination found on the screens. This undoubtedly did not occur directly but indirectly as the animal walked on the screen and then licked its paws in "making its toilet." To one who has observed the habits of rats this possibility is clearly evident.

TABLE I.

Treatment of animals.	Lot 2632.			Lot 2633.		
	Rat No.	Ash.		Rat No.	Ash.	
		Femur.	Humerus.		Femur.	Humerus.
		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
Irradiated.....{	9941	53.2	53.4	9947	52.2	51.9
	9942	52.7	53.3	9948	52.2	53.5
	Average			Average		
	53.1			52.5		
Non-irradiated, kept with irradiated.....{	9939	54.5	54.0	9945	51.8	52.6
	9940	51.6	51.5	9946	53.7	53.8
	Average			Average		
	52.9			53.0		
Non-irradiated controls. {	9943	47.2	46.4	9949	43.9	44.8
	9944	48.8	50.9	9950	45.2	46.5
	Average			Average		
	48.6			45.1		

Bone Analysis.—As already stated the bones of all animals used in the preceding experiments were analyzed for ash upon the termination of the experiments. A humerus and femur were removed from each animal, dissected free from adhering tissue, then dried, and thoroughly extracted with hot alcohol in a Soxhlet extractor. They were ashed in a gas muffle furnace.

As the presentation of the data *in toto* would take up too much space, only the data of the first experiment are presented in their entirety (see Table I). All the data are summarized in Table II.

TABLE II.

Lot No.	Treatment of animals.	Ash.
		<i>per cent</i>
2632	Rats irradiated.	53.1
	Not irradiated, but kept in cage with irradiated.	52.9
	Non-irradiated controls.	48.6
2633	Rats irradiated.	52.5
	Not irradiated, but kept in cage with irradiated.	53.0
	Non-irradiated controls.	45.1
2497	Rats irradiated.	48.9
	Screen irradiated in jar.	49.2
	Empty jar irradiated.	45.1
2520	Rats irradiated.	50.5
	Screen irradiated in jar.	50.2
	Empty jar irradiated.	46.2
2714	Rats irradiated.	55.1
	Copper screen irradiated.	54.2
	Empty jar irradiated.	48.5
2532	Rats irradiated.	51.9
	Grounded screen irradiated.	52.0
	Empty jar irradiated.	43.7
2531	Rats irradiated.	49.5
	Galvanized iron can used as cage irradiated and kept insulated.	49.9
	Galvanized iron can used as cage grounded during irradiation.	50.7
2642	Rats irradiated.	55.5
	Rats given irradiated air.	49.6
	Empty jar irradiated, air blown out.	48.2
2651	Rats irradiated.	54.6
	Granulated zinc irradiated in quartz flask, kept in jar.	48.3
	“ “ “ “ Jena “ “ “ “	47.0
	Irradiated “ put in quartz flask, kept in jar.	48.5
	“ “ “ “ Jena “ “ “ “	48.2
	No irradiation.	48.2

TABLE II—*Concluded.*

Lot No.	Treatment of animals.	Ash. per cent
2787	Rats irradiated.	53.2
	Used screen in quartz flask, irradiated.	49.5
	“ “ “ Jena “ “	49.0
	New “ “ quartz “ “	49.2
	“ “ “ Jena “ “	50.8
	No irradiation.	48.6
2786	Rats irradiated.	53.1
	Screen irradiated; new screen before each irradiation.	44.8
	No irradiation.	44.8
2771	Rats irradiated.	53.3
	Irradiated screen placed above animals.	45.9
	No irradiation.	45.8
2861	Screen irradiated in jar. Jar cleaned, but not the screen.	50.4
	“ 6 inches above and out of reach of animals irradiated.	47.3
	8 inch watch-glass below screen irradiated.	46.1
	Screen washed with hot water before irradiation.	48.1
	“ “ “ “ “ and Gold Dust before irradiation.	46.4
	No irradiation.	47.8
2905	Uncleaned screen irradiated.	54.4
	Screen irradiated after washing with hot water and Gold Dust.	47.7
	No irradiation.	46.9
2906	Uncleaned screen irradiated.	53.7
	Screen irradiated after washing with hot water and Gold Dust.	47.8
	No irradiation.	48.6

Each value given represents the average of two animals for both humerus and femur. To average these appeared entirely defensible, because the results were all as uniform as those already shown in Table I. Taking the values presented, there are apparently no discrepancies between such conclusions as may be drawn from them and those already drawn from the growth curves.

Effect of Irradiated Screens on the Prevention of Rickets.—In our experiments on the prevention of rickets by means of irradiated

screens, we made use of a ration composed of wheat 33, corn 33, wheat gluten 15, gelatin 15, calcium carbonate 3, and sodium chloride 1. This ration has already been used by McCollum and coworkers (10) as Ration 3143 in his work on rickets. Although not specified by him, we always used yellow corn in order to lengthen the lives of our animals by furnishing them with more vitamin A.

In order to obtain maximum effects of any antirachitic action of an irradiated screen we made use of the prophylactic type of experiments rather than the curative. In fact in some preliminary experiments, where we attempted to use the latter to demonstrate calcium deposition by screen effect, early exhaustion of vitamin A brought a premature end to our experiments. In our final trials we used four litters of rats. In the first experiment a litter of six rats, weighing approximately 50 gm. each, was put upon the experimental ration. After a preliminary period of 7 days they were segregated in glass jars provided with false screened bottoms and a record was kept of all food consumed. After a second period of 7 days two rats were directly irradiated. With two others the screen was irradiated and the remaining two were kept as untreated controls. After 2 weeks of this régime the animals were killed and the distal ends of their radii and ulnæ split with a scalpel and stained with silver.

Bones from the untreated control animals showed thickened ends with a typical wide rachitic metaphysis. Those taken from rats on the irradiated screen were normal in one rat and slightly widened in the other. The irradiated controls were entirely normal. It has been our experience that rats vary considerably in the development or repair of the rachitic lesion, even with rats taken from the same litter. For this reason the slight persistence of the lesion in one of the above animals on the irradiated screen is not to be taken as an irregularity of significance. Consumption of the ration during the experiment was entirely satisfactory. The average intake during the last few days of the experiment was in no case appreciably less than 5.2 gm., the average daily consumption of the six rats for the whole period. Therefore, such results as were obtained cannot have been due to deficient consumption of ration.

In the next experiment an attempt was made to demonstrate the positive effect with an irradiated screen placed near, but out

of reach of, the animals. This experiment was carried out essentially in the same manner as before, but to demonstrate the screen effect two concentric cylinders of screen were placed in a jar—the outer in contact with its wall and the inner, $1\frac{1}{4}$ inches removed from it. The rats were confined in the inner cylinder. The outer cylinder was removed twice daily and irradiated 10 minutes, 6 days of the week. Two litters of rats of about the same weight as before were used in this experiment. There was no important deviation from the average food consumption of individual animals at any time.

At the end of the 4th week the experiment was discontinued and the bones of the rats were examined for rickets as before. The examination showed that bones, taken from the irradiated animals, were entirely normal, but those, taken from the untreated, and those, taken from the group in proximity to the irradiated screen, were uniformly rachitic.

It is evident that neither these experiments dealing with the prevention of rickets directly nor those dependent upon growth offer any evidence that irradiated air is physiologically active. We do not, however, desire to put ourselves in the position where we unqualifiedly maintain that air irradiated with ultra-violet light may not have acquired certain special properties acting in this capacity, but we do believe that in the first place there is *a priori* no reason for assuming that it should have, and in the second place the evidence so far presented in its favor is inconclusive. Webster and Hill (11) have already denied the justifiability of Hume and Smith's (4) conclusion, but the irregularity of their data, indicating insufficient experimental control, leaves their arguments unconvincing. We believe that final evaluation of the situation rests with Hume and Smith.

SUMMARY.

Confirmatory evidence is offered that irradiated rats put in the same cage with non-irradiated rats are able to induce growth in the latter even when kept on screens to minimize consumption of excreta.

Evidence is also presented that mere irradiation of the false screen bottoms of rat cages is sufficient to induce normal growth in rats when they are kept on a ration deficient in the antirachitic vitamin.

No evidence was obtained that the above mentioned reactions were brought about by secondary radiations acting externally upon the animals.

No evidence was obtained that irradiation with ultra-violet light will confer growth-promoting or antirachitic properties on air.

It is suggested that the antirachitic growth-promoting reaction was induced in our animals by the ingestion of photochemically activated constituents of the excreta.

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METHEMOGLOBIN.

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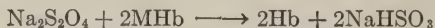
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I. Quantitative Reduction of Methemoglobin to Hemoglobin.

In a previous paper (1) it was shown that the change from reduced hemoglobin to methemoglobin and *vice versa* could be followed electrometrically. The amount of sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$) necessary to reduce methemoglobin and the amount of ferricyanide necessary to oxidize reduced hemoglobin were determined by electrometric titration and compared with the hemoglobin content calculated from a total nitrogen analysis. The results clearly indicated that the change from methemoglobin to reduced hemoglobin involved one hydrogen equivalent of reducing agent. We have now further established this relationship by comparing the results of the electrometric titrations with the methemoglobin content as determined by the method of Van Slyke and Stadie.

This method consists in determining the oxygen capacity of the solution by the well known Van Slyke procedure (2, 3) and the total hemoglobin content by Stadie's modification (4) of the colorimetric cyanhemoglobin method. The difference between the content of total hemoglobin and reduced hemoglobin as given by the oxygen capacity is obviously equal to the methemoglobin content (provided no decomposition of the hemoglobin has occurred); a comparison of the amount of methemoglobin thus determined and the amount of sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$) required in an electrometric titration gives directly the equivalents of reducing agent involved in the titration. The results are summarized in Table I, and an inspection of the last column shows that 0.5 mol of sodium hyposulfite per mol of hemoglobin or

one hydrogen equivalent is required in the reaction:



The last three results obtained with another powerful reducing agent, sodium anthrahydroquinone-2, 6-disulfonate, are of interest as showing that the reaction is a general one and not dependent on any peculiar reaction of the hyposulfite.

It is important to note that while the results are tabulated in terms of mols of hemoglobin, they are really independent of the molecular weight of the compound. The determinations of the amount of hemoglobin and total hemoglobin really rest on the oxygen capacity and are converted to grams of Hb by assuming a molecular weight (16,700) and the formula HbO_2 for oxyhemoglobin; this same value for the molecular weight is used in calculating the molality of the hemoglobin solution so that it cancels in the final result. The values in the next to the last column of Table I are therefore independent of what value is taken for the molecular weight of hemoglobin or methemoglobin, and are based solely on the assumption that 1 mol of hemoglobin combines with 1 mol of oxygen when equilibrated with air. Another way of stating the results of our experiment, therefore, is as follows: *the amount of reducing agent required to transform a given amount of methemoglobin to hemoglobin is one-fourth of that equivalent to the oxygen capacity of the hemoglobin formed by the reduction.* Since Peters (5) has shown that the ratio of iron to combined oxygen in oxyhemoglobin corresponds to 2 atoms of oxygen to 1 of iron, the transformation of methemoglobin to hemoglobin involves *one equivalent of reducing agent per atom of iron.*

The experimental procedure for the electrometric titration was similar to that described in our earlier paper except that a general method of operating was developed which could be employed with solutions containing mixtures of reduced hemoglobin and methemoglobin. The titration vessel of 50 cc. capacity was provided with a mechanical stirrer, a nitrogen inlet and water-sealed outlet, a bright platinum and platinized platinum electrode, a siphon to a saturated calomel electrode, and a burette-tip inlet. Pure atmospheric nitrogen was obtained by passing commercial compressed gas through three Friedrich wash bottles containing alkaline anthrahydroquinone sulfonic acid-sodium hyposulfite solution. A rapid stream of gas so purified was without effect on the

TABLE I.

Comparison of the Electrometric Titrations of Methemoglobin and Its Determination by the Method of Van Slyke and Stadie.

Solution.	$K_3Fe(CN)_6$ per 10 cc. of Hb solution.	$Na_2S_2O_4$ required per 10 cc.	Molality of $Na_2S_2O_4 \times 10^3$	$Na_2S_2O_4$ required, $\times 10^5$	Total Hb (Stadie).	Hb (Van Slyke).	MHb by difference.	MHb mols per 10 cc. $\times 10^5$	Ratio mols $Na_2S_2O_4$ to mols MHb.
	cc.	cc.		mols	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.		
F-1	{ 0.10	4.4	6.10	2.68	13.79	4.91	8.88	5.31	0.495
		4.5	5.85	2.64				5.31	0.497
F-1	{ 0.13	5.1	5.80	2.96	13.79	3.77	10.02	6.00	0.494
		5.1	5.65	2.88				6.00	0.480
F-3	{ 0.12	4.55	6.45	2.94	13.00	3.58	9.42	5.64	0.521
G-1	{ 0.10	7.14	4.50	3.21	13.78	4.27	9.51	5.70	0.545
		7.26	4.48	3.26		4.28	9.50	5.70	0.554
G-1	{ 0.13	10.58	3.27	3.46	13.78	2.59	11.19	6.70	0.516
		13.10	2.65	3.47				6.70	0.517
G-1	{ 0.15	11.99	3.44	4.13	13.77	1.33	12.45	7.45	0.554
		12.11	3.37	4.08				7.45	0.549
G-2	{ 0.08	7.39	4.10	3.03	14.11	4.18	9.93	5.94	0.485
G-2	{ 0.10	8.27	4.25	3.52	14.19	3.76	10.43	6.25	0.564
		8.31	4.14	3.44	14.11	3.70	10.41	6.25	0.550
G-2	{ 0.10	8.87	4.07	3.61	14.12	3.16	10.96	6.56	0.550
		8.70	4.02	3.50				6.56	0.533
G-2	{ 0.10	9.02	3.64	3.28	14.12	3.64	10.48	6.34	0.518
G-2	{ 0.13	10.01	4.23	4.23	14.12	1.21	12.91	7.74	0.546
		10.22	4.07	4.16		1.32	12.90	7.74	0.539
G-2	0.13	10.61	3.69	3.92	14.19	1.25	12.94	7.75	0.506
Using anthrahydroquinone disulfonate as the reducing agent instead of $Na_2S_2O_4$; headings of columns to be read accordingly.									
G-2	{ 0.10	8.3	4.30	3.57	14.12	2.80	11.32	6.80	0.525
		7.98	4.13	3.30				6.80	0.485
G-2	0.13	15.64	2.57	4.03	14.12	1.56	12.56	7.52	0.535

potential of a mixture of indigo disulfonate and its reduction product or upon the color of the completely reduced solution. The solutions of hemoglobin employed were prepared by laking the corpuscular cream of horse blood with about twice the volume of water and centrifuging to remove the small amount of sediment; different samples of corpuscular cream are denoted by letters, and successive lakings, by numbers, throughout this paper. In the experiments listed in Table I, varying proportions of the total hemoglobin were converted to methemoglobin by adding small amounts of saturated ferrieyanide solution to 30 cc. of the hemoglobin solution contained in a tonometer. After evacuation and shaking for 5 minutes to insure complete reaction, a sample of known volume was withdrawn, equilibrated with the oxygen of the air by shaking in a tonometer for 15 minutes, and the oxygen capacity then determined in a Van Slyke apparatus (2). The remainder of the solution was then completely freed from oxygen by evacuating the tonometer and filling with nitrogen and repeating this process eight to ten times. A 10 cc. sample was then pipetted from the tip of the tonometer and quickly transferred to the titration vessel which contained 10 cc. of a phosphate buffer solution (pH 6.8) and had previously been swept out with a rapid stream of nitrogen for 10 minutes. The stream of nitrogen was continued for 5 to 10 minutes more and the electrometric titration with sodium hyposulfite was then commenced. The sodium hyposulfite solution (about 0.01 N) was prepared before a determination by dissolving 0.2 gm. of the solid in 100 cc. of water, containing 0.5 gm. of sodium carbonate. It was protected in the burette with a layer of xylene and it was standardized by rapidly titrating electrometrically a 10 cc. sample of 0.01 N $\text{K}_3\text{Fe}(\text{CN})_6$ solution immediately after each determination. Since a standardization could be completed in 10 minutes after the hemoglobin titration, an error due to the deterioration of the solution was not serious, although it was found that the reducing equivalent of the hyposulfite solution so prepared fell off about 3 per cent per hour.

Since in these experiments we were not interested in the potentials during the titration but only the change of E.M.F. corresponding to the end-point, it was found best to add small increments of reducing agent (about 0.2 cc.) rather rapidly, noting the potential after each addition and continuing the addition of hyposulfite until an increment of 0.1 to 0.2 cc. produced a change in potential of 100 to 200 millivolts (as compared to changes before the end-point was reached of about 3 to 15 millivolts per 0.2 cc.). This large change often occurs very abruptly and there is considerable danger of running by the end-point. This and the incomplete elimination of oxygen are the chief possibilities of error in the titration and the care necessary to avoid them makes the electrometric

determination of methemoglobin a not very satisfactory analytical procedure as compared with Stadie's method and the one given in the following paper. It should be mentioned in this connection that the electrometric titration of reduced hemoglobin with potassium ferricyanide (*cf.* previous paper (1)) is even more difficult to carry out with accuracy and is therefore unsuitable as an analytical method.

II. Oxidation-Reduction Potential of the Hemoglobin-Methemoglobin System.

The rapidity of both the reduction of methemoglobin and the oxidation of reduced hemoglobin, together with the potentials observed during the electrometric titrations and recorded in the previous paper, make it extremely probable that the change is a reversible one governed by the potentials of the hemoglobin-methemoglobin system and that of the oxidizing or reducing agent employed. It has been pointed out in other papers from this laboratory (6) that it is of great importance to establish whether a change involving oxidation or reduction is reversible or essentially irreversible; in the case of a reversible system, such as the ferri- and ferrocyanides, one can define and measure a real oxidation-reduction potential which determines the behavior of the system; on the other hand, irreversible reductions (such as nitrobenzene to aniline) or irreversible oxidations (such as sulfurous to sulfuric acid) cannot be treated in this manner. If the potential of an inert electrode immersed in mixtures containing varying proportions of the two substances is reproducible and conforms to the usual electrochemical equation, one can be certain that the change in question is reversible. We have now examined mixtures of hemoglobin and methemoglobin from this point of view and while the results leave much to be desired they afford additional evidence of the reversibility of the oxidation and reduction processes involving these substances. The results also serve to evaluate the oxidation-reduction potential of the system and it is gratifying to find them in general agreement with those obtained by the titration method.

The methemoglobin employed was prepared in the usual manner (7). Corpuscular cream from fresh horse blood, thrice washed with hypertonic salt solution (1.5 per cent), was laked with the least possible amount

of water. To 500 cc. of the warm concentrated solution, 1.5 gm. of potassium ferricyanide were added and the solution was stirred until the reaction had become complete. The dark solution was cooled to 0° and 0.25 volume of 95 per cent alcohol, also cooled to 0°, was added with vigorous stirring. After standing for 2 days in a well packed salt-ice mixture, a copious crop of minute crystals had appeared. These were separated from the mother liquor by centrifuging and immediately dissolved in distilled water at 37.5°. The crystallization was repeated and the thick paste separated by the centrifuge was dissolved in water without further removal of mother liquor by filtration. The solution so obtained (400 cc.) was clear reddish brown in color and no solid residue was detected on filtering. It undoubtedly contained small amounts of ferricyanide or its reduction product and of alcohol; these impurities had no noticeable influence, however, and presumably would not influence our results.

The concentration of the methemoglobin solution was determined by a method which is given in detail in the following paper and the validity of which has been carefully demonstrated. The solution employed contained 6.15 (± 0.02) per cent of methemoglobin. Carefully measured amounts of this solution and a 10.66 (± 0.05) per cent solution of freshly prepared hemoglobin solution (from the same horse blood) were mixed in such proportions that three solutions resulted, having the following composition.

Solution.	Hemoglobin content.	Methemoglobin content.
	<i>mols per l.</i>	<i>mols per l.</i>
A	1.06×10^{-3}	3.07×10^{-3}
B	2.12×10^{-3}	2.45×10^{-3}
C	4.24×10^{-3}	1.23×10^{-3}

Neither the hemoglobin nor methemoglobin solutions were purified by dialysis and therefore undoubtedly contained certain amounts of salts, but since the potential measurements were made with heavily buffered solutions, the presence of these salts can be neglected.

Very concentrated buffer solutions were prepared and their pH determined after dilution with sufficient distilled water to give a total salt concentration of 0.1 M. These measurements, as well as those to follow, were made with a saturated calomel electrode whose potential at 25° was found to be 0.2490 by comparison with a carefully prepared 0.1 N calomel electrode and with

a hydrogen electrode containing 0.05 M potassium acid phthalate. Sufficient amounts of these concentrated solutions were added to mixtures A, B, and C to give a total salt concentration of 0.1 M, and it was assumed that the pH values of the resulting solutions were the same as those of the pure buffer solutions of equal dilution. The concentrations, compositions, and amounts added to 30 cc. of hemoglobin-methemoglobin mixture were as follows:

Composition.	pH	Concentration.	Added to 30 cc. of hemo- globin solution.
		N	cc.
$\text{KH}_2\text{PO}_4 + \text{NaHPO}_4$	6.42	2.5	1.25
$\text{H}_3\text{BO}_3 + \text{borax}$	8.44	0.833	4.20
$\text{H}_3\text{BO}_3 + \text{NaOH}$	9.45	1.306	2.30

In carrying out an experiment 30 cc. of a given mixture (A or B or C) were measured into a tonometer, the buffer solution was added, and the tonometer tightly sealed with a rubber stopper, coated with collodion. After evacuating and filling with pure nitrogen ten times, the tip of the tonometer was connected with a tube projecting through the stopper of a $\frac{1}{2}$ oz. bottle which served as the electrode vessel. It was equipped with a sealed nitrogen outlet, a bright and a gold-plated platinum electrode, and a siphon tube containing saturated potassium chloride solution. The side tube of the tonometer was connected with the nitrogen supply and the stop-cock turned so as to connect the side tube with the tip and allow nitrogen to enter the electrode vessel through the tube which extended to the bottom of the bottle. A second similarly equipped vessel was connected in series with the first. After sweeping out the two bottles with a rapid stream of nitrogen for $\frac{1}{2}$ to 1 hour, half of the solution was run into the first vessel under a pressure of nitrogen, which could be regenerated by placing the vessel again in connection with the nitrogen supply. The inlet and outlet tubes were then closed with pinch-cocks, the tonometer was removed and carefully connected with the second vessel without introducing any air. After a few minutes additional sweeping, this bottle was filled with solution. In a few cases nitrogen was allowed to bubble through the solution in one of the bottles for 10 to 15 minutes but no difference in the potentials due to this additional precaution could be detected.

Measurements were made at $25 \pm 0.05^\circ$ against the saturated calomel electrode. The potentials on both the bright platinum and the gold-plated electrodes were read every 5 to 10 minutes for periods varying from $\frac{1}{2}$ to 2 hours. In this period of time a fair degree of constancy had been reached in each case. The first few readings were usually erratic and were discarded. After that the potential in some cases became quite

constant, the readings on both electrodes agreed, and were not altered by vigorous shaking; in other cases the constancy and agreement were not so good and shaking altered the potential. In the latter event, however, it was perfectly apparent when the initial "drift," due, presumably, to the establishment of equilibrium at the liquid junction and to the attainment of the temperature of the thermostat, had ceased and when no further change was taking place. Consequently, from five to ten readings on each electrode for the quiet and the shaken solution were recorded at regular intervals during the period of this state of relative constancy and the average of these was taken as the potential of the cell. Thus, for example, the following cell potentials were recorded in Experiment 18.

Time.	π (Gold).	π (Pt).	Remarks.
<i>min.</i>			
Discarded { 4	-0.157	-0.158	Shaken.
5	-0.155	-0.167	"
10	-0.150	-0.155	Quiet.
	-0.153	-0.158	Shaken.
20	-0.145	-0.156	Quiet.
	-0.148	-0.159	Shaken.
30	-0.139	-0.156	Quiet.
	-0.144	-0.163	Shaken.
40	-0.143	-0.150	Quiet.
	-0.149	-0.156	Shaken.
50	-0.139	-0.155	Quiet.
	-0.144	-0.155	Shaken.

The results of experiments with the different hemoglobin-methemoglobin mixtures in buffer solutions of three different pH values are summarized in Table II. The potentials are all referred to the normal hydrogen electrode. In order to indicate the behavior in each case, the highest and lowest potentials observed with each electrode have been recorded. In the next to the last column are given the values for π_n calculated according to the following equation in which the value of the last term (given in the fourth column of the table) was calculated from the known composition of the mixture of hemoglobin and methemoglobin employed.

considered to be in agreement with the electrochemical equation. Thus, with the exception of the results with A in pH 8.44, the potentials in each buffer are in the order $A > B > C$, as predicted from the equation. The potential of a given mixture in a particular buffer seems to be fairly reproducible, the average divergence

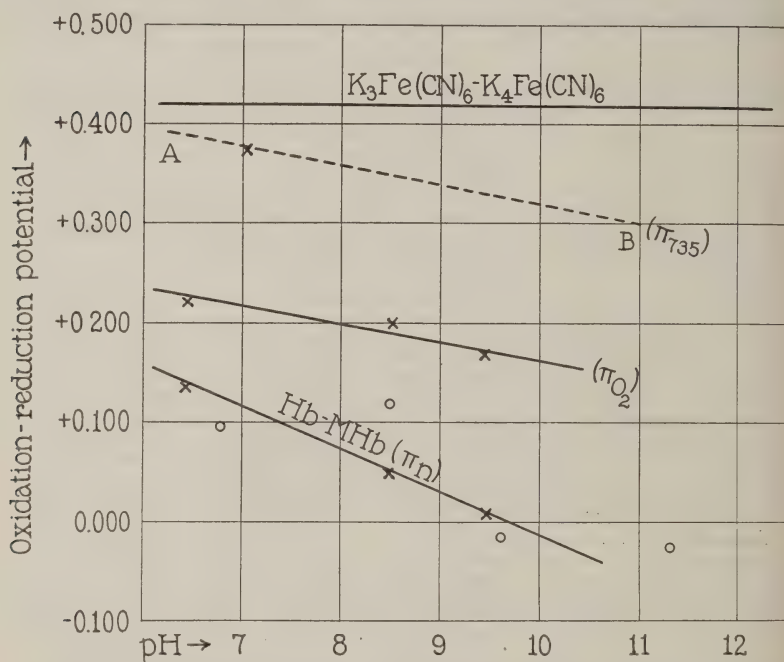


FIG. 1. Oxidation-reduction potentials of $K_3Fe(CN)_6$ - $K_4Fe(CN)_6$ and Hb-MHb. The curves showing the changes of normal oxidation-reduction potential with hydrogen ion concentration are as marked; the crosses represent experimental points given in this paper, the circles, previous determinations by the titration method. The broken line AB is the estimated true Hb-MHb potential curve at 735 mm. of O_2 ; the curve π_{O_2} gives the observed potentials of Hb-MHb mixtures saturated with oxygen.

being 8.3 millivolts and the maximum, 22 millivolts. Considering the complexity of the hemoglobin molecule and the necessarily low *molecular* concentration of the solutions, the results are probably as satisfactory as could be expected.

In general the results of this determination of the oxidation-reduction potential by the method of mixtures are in agreement

with the values given in the first paper (1) which were obtained by the titration method. This is illustrated by Fig. 1 in which the present determinations and the earlier ones are both plotted. In one respect it is necessary to modify the earlier views; the sharp drop in potential between pH 8.5 and 9.8, which was indicated by the first measurements, has not been confirmed. Instead, the change of potential with change in pH seems to extend gradually over the whole range. Not too much significance can be attached to the exact magnitude of this change, however, since the activity of the hemoglobin and methemoglobin is undoubtedly greatly affected by the salt concentration as well as by the hydrogen ion concentration and in our present work no data are available to enable us to distinguish between these two effects of the buffer solution. Thus, while we believe that the oxidation-reduction potential of methemoglobin in any solution of definite pH is probably fairly close to the value corresponding to the curve in Fig. 1, the exact slope of this particular curve is probably not very significant. The general downward trend indicates that methemoglobin is a weaker acid than hemoglobin, but instead of only 1 acidic hydrogen atom being involved as first suggested¹ there are probably several acid groups functioning in this range.

Passing oxygen or carbon monoxide into a mixture of hemoglobin and methemoglobin was shown in the previous paper (1) to increase the oxidation-reduction potential. The reason is evident from a consideration of equation (1); if the term $[Hb]$ is greatly diminished by combination of the hemoglobin with the gas (formation of oxy- or carboxyhemoglobin) and $[MHb]$ stays unchanged, the potential will increase. Hemoglobin combines with either oxygen or carbon monoxide, methemoglobin does not; hence, saturating the solution with one of these gases changes the potential greatly. The values given in the last column of Table II (π_{O_2}) were obtained by passing oxygen into the electrode vessel after the determinations in the absence of oxygen were complete. The vessels were shaken and the flow of oxygen was continued until a constant potential was reached which is recorded in the table.

The position of these potentials is indicated in Fig. 1. Undoubtedly these values are much lower than the true potential

¹ Conant (1), p. 411.

of an equimolecular mixture of hemoglobin and methemoglobin equilibrated with oxygen, since the amount of unoxygenated hemoglobin is very small in a solution saturated with oxygen (too small to be appreciable in an analytical procedure) and the potential of an oxidized compound containing traces of the reduced compound is never as high as that predicted from the electrochemical equation. The equilibrium at the surface of the inert electrode is probably established with great difficulty after the concentration of one of the components reaches a certain small value. These observed potentials are, therefore, merely indicative of the effect of removing nearly all the reduced compound (the hemoglobin) from the system by causing it to combine with oxygen. It is probable that in each buffer solution saturated with oxygen the potential of the electrode is some constant fraction of the very high true potential of the system. If this is so, a line parallel to the π_{O_2} line, but much higher, would correspond to the real potential of such an almost completely oxygenated system; evidence is given below which leads us to place such true potentials in the position indicated in Fig. 1 by the broken line AB .

It is interesting to note that the difference between π_n and π_{O_2} is greater the more alkaline the solution. This must mean that the more alkaline the solution the greater the fraction of hemoglobin which unites with oxygen at a given pressure. This increase in the affinity of hemoglobin for oxygen with increased alkalinity has been deduced from entirely other evidence (8), and the fact that our results lead to the same conclusions is further evidence of the correctness of our general interpretation.

III. Action of Ferricyanide on Hemoglobin Solutions.

Both of the systems, ferricyanide-ferrocyanide and hemoglobin-methemoglobin, have real oxidation-reduction potentials and the extent to which these systems will interact must be determined by the value of these potentials. A glance at Fig. 1 makes it evident that reduced hemoglobin, *in the absence of any gas with which it combines*, will be practically completely oxidized by one equivalent of ferricyanide, since the difference in potential between the systems is some 250 millivolts. This has been found to be the case by electrometric titration (1). If, however, the two systems interact in the presence of oxygen, it is not the potential of equi-

molecular mixtures of hemoglobin and methemoglobin which determines the reaction, but the potential of a solution saturated with oxygen at the particular pressure employed. We have mentioned above that curves representing such potentials for relatively high oxygen tensions would presumably parallel the π_{O_2} line in Fig. 1 and lie far above it; if such a curve lay close enough to the ferricyanide-ferrocyanide potential, the reaction between equimolecular amounts would not go to completion, but to a definite equilibrium. Such we have now found to be the case.

TABLE III.

Extent of Oxidation of Hemoglobin by Varying Amounts of $K_3Fe(CN)_6$ in Presence of O_2 (735 Mm.) ($t = 25 \pm 3^\circ$).

Initial Hb concentration.	$K_3Fe(CN)_6$		Final Hb concentra- tion.	Fraction of Hb converted into MHb. (\bar{R})	K'
	<i>gm. per 100 cc.</i>	<i>equivalents</i>	<i>gm. per 100 cc.</i>		
	0.138	0.50	8.89	0.365	0.64
	0.200	0.73	6.25	0.554	0.26
14.16	0.276	1.00	3.44	0.754	0.11
13.78	0.300	1.09	2.91	0.792	0.10
14.10	0.368	1.33	2.30	0.837	0.12
14.01	0.368	1.33	2.46	0.826	0.09
	0.552	2.00	1.77	0.875	0.11
	1.104	4.00	1.51	0.892	0.27
(By extrapolation, 4.1.....13.5 for1.00)					
Average*.....					0.15

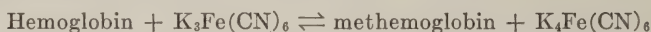
The Hb content was determined in the usual way from the oxygen capacity as measured in the Van Slyke apparatus; the solutions were in every case equilibrated with oxygen and the apparent oxygen capacity corrected for dissolved oxygen at 735 mm. of pressure.

* Omitting the first value.

A neutral solution of hemoglobin treated with 1 mol of potassium ferricyanide and then equilibrated with oxygen at about 735 mm. still liberates about one-quarter of the original amount of bound oxygen when analyzed in the Van Slyke apparatus. It should be noted that in the Van Slyke procedure the reaction with ferricyanide liberates the oxygen in a vacuum and at the resulting low partial pressure of oxygen the reaction runs to completion because the hemoglobin potential under these conditions is not very different from the normal potential in an inert atmosphere.

A series of experiments which illustrates this behavior is summarized in Table III. A fresh solution (I-1) of hemolyzed, unwashed, fresh corpuscular cream (horse) was analyzed with the results shown in the first column. A definite amount of solid $K_3Fe(CN)_6$ was added to a 10 cc. sample of this solution and the latter was then shaken in an evacuated tonometer for 3 minutes. Oxygen was then introduced and the solution analyzed after shaking for 15 to 20 seconds. The number of equivalents of ferricyanide was calculated from the average analysis of the fresh solution and the percentage of the material oxidized was computed from this value, together with the concentration of hemoglobin found after the treatment described. The temperature was about 25° . From the table it will be seen that four equivalents of ferricyanide failed to reduce the oxygen capacity to zero and that about 13.5 equivalents would probably be required for complete oxidation *under the conditions of these experiments*.

If the reaction



be considered as a reversible reaction coming to equilibrium rapidly, the value of the equilibrium constant may be expressed as follows:

$$(2) \quad K = \frac{[K_3Fe(CN)_6] [Hb]}{[K_4Fe(CN)_6] [MHb]}$$

Since at equilibrium,

$$[K_3Fe(CN)_6] = [K_3Fe(CN)_6]_i - [MHb]$$

$$[K_4Fe(CN)_6] = [MHb]$$

$$[Hb] = ([Hb]_i - [MHb]) F$$

$$[MHb] = R [Hb]_i$$

if E = equivalents of $K_3Fe(CN)_6$ per mol of hemoglobin, R = the fraction of Hb oxidized to MHb, F = the fraction of total reduced hemoglobin (Hb) present as such after equilibrating with oxygen, and $[]_i$, the initial concentrations of the reactants, we may write, by substituting in equation (2),

$$K = \frac{(E [Hb]_i - R [Hb]_i) ([Hb]_i - R [Hb]_i) F}{R^2 [Hb]_i^2}$$

Or,

$$(3) \quad K = \frac{(E - R) (1 - R) F}{R^2}$$

With a given hemoglobin solution, a definite oxygen tension, and temperature, F becomes constant and we may write

$$(4) \quad K' = \frac{K}{F} = \frac{(E - R)(1 - R)}{R^2}$$

The values of this inclusive constant, K' , have been calculated according to this equation and are included in Table III. With the exception of the first experiment, there is a fair approximation in the values to an average of 0.15. From K' we can readily calculate the oxidation-reduction potential of a system composed of equimolecular amounts of hemoglobin and methemoglobin saturated with oxygen at 25°. It should be noted, in the experiments recorded in Table III, that while the ferricyanide was added to the hemoglobin *in vacuo*, the final equilibration was performed with oxygen at atmospheric pressure (about 735 mm; *i.e.*, 760 minus the aqueous tension). The reaction undoubtedly went practically to completion when the ferricyanide was first added, but on shaking with oxygen reversed to a certain degree as the hemoglobin was converted to oxyhemoglobin; the value of K' , therefore, corresponds to the true oxidation-reduction potential of hemoglobin-methemoglobin saturated with oxygen at 735 mm. and which we were unable to measure experimentally for the reasons given above. The calculation of this potential, which we shall designate as π_{735} , is as follows:

$$\pi_{735} - \pi_{FC} (\text{normal potential of } K_3\text{Fe}(\text{CN})_6) = 0.059 \log K'$$

since at equilibrium the potential of the two systems are equal and therefore

$$\pi_{FC} + 0.059 \log \frac{[\text{Fe}(\text{CN})_6^{\text{III}}]}{[\text{Fe}(\text{CN})_6^{\text{IV}}]} = \pi_{735} + 0.059 \log \frac{[\text{MHb}]}{[\text{Hb}]_{\text{total}}}$$

or

$$\pi_{735} - \pi_{FC} = 0.059 \log \frac{[\text{Fe}(\text{CN})_6^{\text{III}}] [\text{Hb}]_{\text{total}}}{[\text{Fe}(\text{CN})_6^{\text{IV}}] [\text{MHb}]} = 0.059 \log K'$$

Taking the value 0.420 for the ferricyanide potential (9),

$$\pi_{735} = 0.420 + 0.059 \log K' = 0.372$$

Assuming the pH of this unbuffered solution to be about 7.0, the potential has been plotted in Fig. 1 and through it a broken line

(*A B*), parallel to the observed potentials of the oxygenated hemoglobin mixtures, has been drawn. *This curve, we believe, represents approximately the true oxidation-reduction potential at 735 mm. of pressure of oxygen of the hemoglobin-methemoglobin system, and its position determines the behavior of such a system when treated with oxidizing agents.*

The downward trend of this curve with increasing alkalinity is of importance and is verified by a few experiments we have performed in buffered solutions which are recorded in Table IV. In each experiment the hemoglobin solution employed was prepared by hemolysis of twice washed fresh corpuscular cream (horse). The requisite amount of concentrated buffer solution (previously described) to give a resulting 0.1 M solution was diluted with the hemoglobin solution to 50 cc. After determining the oxygen capacity of this buffered solution, a 10 cc. sample was treated with a definite amount of solid potassium ferricyanide and shaken *in vacuo*. The solution was then equilibrated with oxygen at atmospheric pressure and the oxygen capacity again determined. It will be noted that the amount of oxidation per equivalent of ferricyanide at pH 6.42 is about the same as in the experiments with the unbuffered solution given in Table III. In the more alkaline buffers, however, much more complete oxidation takes place. This would be predicted from a potential curve corresponding to *AB* in Fig. 1; the more alkaline the solution the greater the difference in potential between the ferricyanide potential (which is essentially independent of the pH) and the hemoglobin potential (π_{735}), and the farther the reaction proceeds.

The facts represented graphically in Fig. 1 explain the success of the Haldane method of determining the oxygen capacity of hemoglobin. Although the amount of ferricyanide employed in this analytical procedure usually corresponds to only about two equivalents, the reaction liberates practically all the oxygen because it is carried out either at a very low oxygen pressure (Van Slyke method) or *in alkaline solution*. Thus in the original Haldane procedure (10), sodium carbonate was added, and ammonia has often been similarly employed; in the Van Slyke procedure, sodium hydroxide is often added to absorb the carbon dioxide. The addition of any of these substances would make the solution at least as alkaline as pH 9.5, and under these conditions

the oxidation by ferricyanide is practically complete, even if the reaction is carried out at atmospheric pressure (Table IV and Fig. 1).

The results of a number of previous investigators are entirely in accord with our interpretation of the reaction between hemoglobin and ferricyanide, although as they stand in the literature, they seem to be somewhat inconsistent with one another unless viewed in the light of the present research. Von Zeynek (11) undertook to establish the ratio of potassium ferricyanide to hemoglobin by an elaborate method which, in common with

TABLE IV.

Oxidation of Hemoglobin by Ferricyanide in Buffer Solutions in the Presence of Oxygen at 735 mm. (25°).

Experiment No.	Solution.	pH	Percentage Hb.		K ₃ Fe(CN) ₆ added.	Oxidation.
			Initial.	After adding K ₃ Fe(CN) ₆ .		
					<i>equivalents</i>	<i>per cent</i>
1	J-1	6.42	11.25	2.49	0.992	77.8
2	J-1	6.42	11.25	0.77	4.71	91.2
3	J-2	6.42	9.04	1.99	1.0	77.9
4	K-1	6.42	10.87	3.27	1.0	65.8
5	K-1	6.42	10.87	2.49	2.0	77.2
6	K-1	8.44	11.10	0.98	2.0	91.3
7	K-1	8.44	11.47	0.53	1.17	95.4
8	K-1	8.44	11.47	0.34	1.55	97.2
9	K-1	9.45	11.27	0.14	2.0	99.0

the Haldane method, involved equilibration of a hemoglobin solution with the oxidizing agent in the presence of air. The solution, however, was not alkaline and his failure to liberate the full calculated amount of oxygen is exactly parallel to our experiments under similar conditions. It is customary to quote the extremely accurate measurements of von Reinbold (12) as constituting the best evidence of the ratio of oxyhemoglobin to potassium ferricyanide. While his evidence on this point is indeed quite convincing, one is impressed, on examining his data, with the rather surprising fact that the two methods of analysis, both of which were quite accurate, yielded appreciably different results

with identical hemoglobin solutions. In the first method oxygen was liberated into an atmosphere of hydrogen containing only a little oxygen. After this analysis the sample of the same solution was withdrawn, diluted with water, and examined at leisure at the atmospheric oxygen tension with the spectrophotometer. In ten experiments the first method gave an average value of 0.95 for the ratio in question, while the average by the second method was 0.83. That the value found gasometrically agrees with the theoretical value within the experimental error simply shows that the reaction proceeds to completion at a low oxygen tension. That the other figure, obtained by the optical method, is decidedly too low may be attributed to a reversal of the oxidation in the presence of atmospheric oxygen with the formation of a small amount of oxyhemoglobin. Indeed, von Reinbold states that the absorption bands of this substance were always discernible. Wertheimer (13) compared the oxygen capacity of solutions with their iron analyses, making use of the Bancroft-Haldane method. His results agreed with the theory within 1.5 per cent when soda-alkaline solutions were used, but were 7 per cent lower when pure neutral solutions were employed. He pointed out that either there must be more oxygen bound in alkaline solutions, which is unlikely, or else ferricyanide liberated the full amount only in alkaline solutions. The obvious interpretation from the present point of view is that oxidation was incomplete in neutral solutions because of the high oxygen tension while the presence of alkali increased the difference in the potentials of the two systems and allowed oxidation to proceed to completion. Very recently Meier (14) has studied the reaction between oxyhemoglobin and ferricyanide and quinone and formulated the reaction as a reversible one proceeding to an equilibrium point depending on the oxidizing power of the quinone and ferricyanide. This interpretation differs from ours in that it represents the reaction taking place between the oxidizing agent and the oxyhemoglobin and does not consider the effect of change of hydrogen ion concentration. Meier, using a manometric method, found that one equivalent of ferricyanide liberates only about 80 per cent as much oxygen as an excess of the reagent. Since the gas was liberated in neutral solution at atmospheric pressure, this result is in accordance with our experiments, although Meier hesitated to draw any conclusion

from this one experiment. As we shall show in another paper, we are in general agreement with him as to the reasons for the differences between the action of ferricyanide and quinone.

The position of the line AB (π_{735}) in Fig. 1 and the value of K' (equation (4) and Table III) are obviously dependent on F , the fraction of the reduced hemoglobin which is *uncombined* with the oxygen under the stated conditions (25° and 735 mm.). It is interesting to calculate this value from the normal potential in the absence of oxygen (π_n) and the data in Table III, which have allowed us to evaluate the true potential in the presence of oxygen. (It is immaterial whether we calculate F by means of equation (4) (from K and K') or in a more general manner by reference to the oxidation-reduction potentials of the systems in the presence and absence of oxygen.)

From equation (1) and the relation $[\text{Hb}] = F [\text{total Hb}]$, we have,

$$\pi_{735} = \pi_n + 0.059 \log \frac{[\text{MHb}]}{F [\text{total Hb}]}$$

Since by definition

$$[\text{MHb}] = [\text{total Hb}]$$

when $\pi_{\text{observed}} = \pi_n$

$$(5) \quad \pi_{735} = \pi_n - 0.059 \log F$$

Substituting the values at pH = 7.0 for π_{735} and π_n

$$0.059 \log F = -0.270 \quad \log F = -4.5 \quad F = 3 \times 10^{-5}$$

It is of great interest to compare this value for F with that obtained by extrapolation of the data obtained from the usual measurements of the oxygen capacity of hemoglobin solutions at relatively low pressures of oxygen. Hill's equation, $\frac{y}{1-y} = K_1 x^n$, has been found to express the relationship between the degree of oxygen saturation (y) and the pressure (x) quite satisfactorily (15), K_1 being a constant and n being an integral number. In order to evaluate K_1 , it is convenient to note that

$$(6) \quad \log \frac{y}{1-y} = \log K_1 + n \log x$$

and, therefore, when $y = 0.5$

$$(7) \quad \log K_1 = -n \log x$$

Taking Ferry's measurements (16) with horse hemoglobin at 38° and assuming a shift in oxygen dissociation curves with changes in temperature corresponding to those observed by Brown and Hill (17) for human

blood² one can estimate that $x = 8$ mm. when $y = 50$ per cent for unbuffered horse hemoglobin at 25°. If $n = 2.5$ (a value Hill has found to hold for a number of cases), $\log K_1 = 2.25$. Of course, this is only a rough approximation, but will serve to show whether our results are of the right order of magnitude as compared with the actual measurements of the oxygen dissociation curves of horse oxyhemoglobin. It is evident that since $F = 1 - y$ and, for very small values of F , $1 - F$ may be taken as equal to 1 without serious error, equation (6) becomes

$$(8) \quad \log \frac{1}{F} = -\log F = \log K_1 + n \log x$$

Substituting -2.25 for $\log K_1$, $n = 2.5$, and $x = 735$, we find $\log F = 4.93$ or F is practically 10^{-5} . The agreement with the value calculated from our measurements (3×10^{-5}) is all that could be expected and is gratifying evidence of the correctness of our interpretations.

In connection with the validity of Hill's equation over a very wide range of oxygen pressures, it is important to note that if $n = 1$, the divergence between F calculated from the dissociation curves and our measurements would be enormous. If the simple formula $\frac{y}{1-y} = K_1$ were a correct expression of the behavior of the oxyhemoglobin-hemoglobin-oxygen system, our results would be quite inexplicable since π_{735} would then be expected to be about 0.1 volt below the value we have found; the ferricyanide reaction then should go to practical completion, even in a neutral solution saturated with oxygen. It is worth while pointing out, perhaps, that measurements like ours of the equilibrium between an oxidizing agent (such as ferricyanide) and hemoglobin under definite conditions enables one to calculate n directly. Combining equations (7) and (8), we have

$$(9) \quad \log F = n (\log x_1 - \log x_2)$$

where x_1 = pressure at 50 per cent saturation and x_2 = pressure at which F represents the fraction uncombined. Solving this with the data just given ($\log F = -4.5$ from our measurements, $x_2 = 8$ mm., estimated from Ferry's results), we find $n = 2.28$. The agreement between this and the values Hill has found for n (from 2.2 to 2.5) is merely another way of showing the entire agreement between these apparently very distantly related observations.

It seems certain from all this evidence that the factors governing the reaction between potassium ferricyanide and hemoglobin are adequately represented by the equations given above and the potential curves in Fig. 1. It should be pointed out, however, that the effects of the presence of salts on the hemoglobin-oxygen

² Brown and Hill (17), Fig. 4.

system and on the hemoglobin-methemoglobin system have been neglected. The presence of considerable amounts of neutral salts is known to influence the oxygen dissociation curves and may influence to some extent the oxidation-reduction potential of hemoglobin; in a more exhaustive and accurate treatment of this subject such influences would have to be taken into account. It may also be that the value of π_n , K , and n vary somewhat with different samples of hemoglobin from the same species. Thus, in Table IV parallel experiments with different hemoglobin solutions from the same corpuscular cream agreed closely (Experiments 1 and 3), but with different material varied more than can be accounted for by our experimental errors (Experiments 3 and 4).

Such divergences may be due to the presence of salts or real differences in the nature of the hemoglobin; further experiments with carefully purified hemoglobin will be necessary in order to settle this point.

The addition of potassium ferrocyanide to the hemoglobin solution should decrease the amount of oxidation caused by a certain amount of ferricyanide. This prediction has been verified qualitatively in two experiments at pH 6.42, but the extent of the effect has not agreed very well with that calculated. For example, in two experiments four equivalents of potassium ferrocyanide were added and the percentage of hemoglobin solution oxidized should have been 55 per cent (as compared with 78 per cent in the absence of ferrocyanide); the actual values were 62.8 and 75.7. The discrepancy is greater than our experimental error and may be due to a salt effect of the very large amount of ferrocyanide necessarily employed.

IV. Structure of Methemoglobin and Hemoglobin.

The relationship between methemoglobin and hemoglobin has been expressed by the following different symbols: $\text{Hb} \begin{array}{c} \diagup \text{O} \\ | \\ \diagdown \text{O} \end{array}$,

HbO , and HbOH , where Hb is the reduced hemoglobin molecule and HbO_2 the oxyhemoglobin molecule. Although for the past 15 years a number of German investigators (in particular Küster and Heubner) have employed the formula HbOH , most texts still

write $\text{Hb} \begin{array}{c} \diagup \text{O} \\ | \\ \diagdown \text{O} \end{array}$ or its equivalent and very recently Roaf and

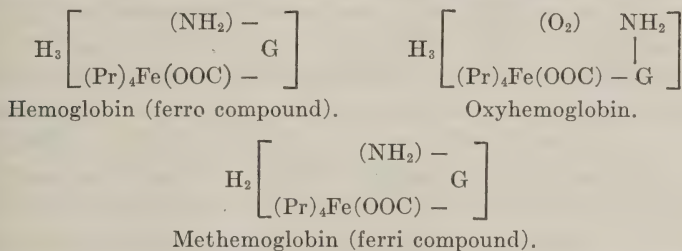
Smart (18) and Quagliariello (19) claim to have established the correctness of the formula HbO .

As Quagliariello has pointed out, there is a mass of evidence already in existence which shows that methemoglobin cannot be represented as having the same amount of oxygen as oxyhemoglobin and probably no one thoroughly acquainted with the field would now support the first of the symbols given above. While all investigators thus agree that methemoglobin has less oxygen than oxyhemoglobin and more than reduced hemoglobin, there is still a great difference of opinion as to whether the formula HbO or HbOH (equivalent to Hb_2O) is correct.

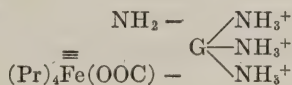
The results presented in this and the previous paper established beyond doubt that *one hydrogen equivalent of oxidizing agent* is used up in converting reduced hemoglobin to methemoglobin and *one hydrogen equivalent of reducing agent*, in bringing about the reverse change. The formula HbOH is in accord with these facts, the formula HbO is not. As Küster (20) pointed out in 1910, methemoglobin is the ferri compound and reduced hemoglobin and oxyhemoglobin are ferro compounds. The results of our electrometric titrations permit of no other interpretation.

However, it seems to us that the symbols HbOH and Hb fail to express adequately the significant facts concerning the relationships of the compounds. Strictly speaking, it is not a question of how much oxygen is bound in methemoglobin,—no oxygen is combined in the molecule any more than any oxygen is combined in ferric chloride or potassium ferricyanide. In passing from reduced hemoglobin to methemoglobin, we are concerned with a change of valence of the iron atom, while, on the other hand, the formation of oxyhemoglobin involves the actual combination of a molecule of oxygen with the pigment. The reversibility of oxidation and reduction processes involving hemoglobin and methemoglobin and the fact that significant oxidation-reduction potentials can be measured are convincing evidence that we are dealing with complex ions differing in valence by one. In modern terminology the gain or loss of one electron from these ions is the fundamental change involved. This might be represented by HbOH if we understood it to mean Hb^+OH^- , but not if we understood the hydroxyl group to be firmly attached to the iron atom as is believed to be the case in hematin.

Both hemoglobin and methemoglobin are amphoteric substances having a number of acidic hydrogen ions; in solutions more alkaline than their isoelectric point they are present as negative ions. If we represent hemoglobin as $H_x(\text{Hb})$, then methemoglobin is $H_{x-1}(\text{Hb})$, and in strongly alkaline solutions where all the hydrogen atoms are involved the ions would be $(\text{Hb})^x$ and $(\text{Hb})^{x-1}$. It was pointed out in the first paper that the analogy between certain aminoferrocyanides and hemoglobins was very striking and on the basis of this analogy the following formulas were written (as the sodium salts, here indicated as the corresponding acids).



The symbol (Pr) stands for the four pyrrole residues found in hematin minus 4 hydrogen atoms (*i.e.* 4 pyrrole ions) and G for the globin molecule of which one carboxyl group and one amino group are indicated. The negative valence of the complex ion would be three in the ferro compound, hemoglobin, as 5 negative ions (4 pyrrole and 1 carboxyl ion) are involved in the complex. Besides the acidic hydrogen atoms which are thus pictured as being necessary consequences of the formation of the complex ion, there are, of course, others in the large globin molecule and probably one or two joined to the pyrrole nuclei. These and the free amino groups of the protein are not represented. In very alkaline solution the value of x in $\text{Na}_x(\text{Hb})$ would be the sum of the 3 hydrogen atoms depicted above and the others just mentioned; at the isoelectric point the 3 hydrogen atoms would be undissociated and probably would be taken up by three amino groups in the protein molecule; isoelectric hemoglobin, from this point of view, would be represented rather by an "inner salt" formula than by that given above. Such a formula might be written:



Similar formulas for isoelectric methemoglobin and oxyhemoglobin are readily written.

Until some method is available for estimating the dissociation constants of the various acidic groups and basic groups it is impossible to predict the isoelectric point from such formulas. However, the well known fact that the very basic globin when combined in the hemoglobin molecule becomes a much more acidic protein (isoelectric point = pH 6.9) is in accord with our point of view. Moreover, as pointed out in the first discussion of these formulas, the marked differences in the strength of hemoglobin and oxyhemoglobin as acids find a reasonable interpretation. The 3 acidic hydrogen atoms which are essential to the complex ion should differ in their degree of dissociation according to the nature of the groups bound to the central atom (iron) of the complex. One could not predict whether the replacement of a coordinately held amino group by an oxygen molecule would increase or decrease the dissociation of the 3 most acidic hydrogen atoms, but it should produce a marked effect. Such differences are well known and can hardly be accounted for in terms of changes in the dissociation constants of the acidic groups of the protein molecule.

Since the structural formulas suggested above are necessarily somewhat uncertain and are, furthermore, cumbersome to employ, we suggested that the ordinary transformations of hemoglobin can be best represented by the following formulas, the symbol MHb being retained merely to remind the reader of the differences between the compounds.

	Acid solution.	Isoelectric.	Alkaline.
Hemoglobin.....	$(H_3HbH)^+$	$H_3(Hb)$	$Na_3(Hb)$
Oxyhemoglobin.....	$(H_3HbO_2H)^+$	$H_3(HbO_2)$	Na_3HbO_2
Methemoglobin.	$(H_2MHbH)^+$	$H_2(MHb)$	Na_2MHb

If Hill's theory (15) of the aggregation of the hemoglobin molecule is the correct explanation of his equation, the above formulas would have to be represented as polymerizing to a certain degree under certain conditions. Such an assumption in no way invalidates the formulas or the general theory developed in this paper. The molecule containing 1 atom of iron still can be

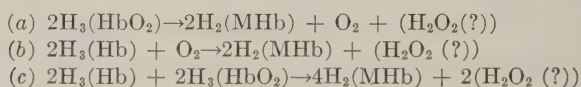
regarded as the essential hemoglobin unit and in our discussion we have been concerned only with the formulation of this ultimate molecule.

The discrepancy between our results and those of Roaf and Smart (18) and Quagliariello (19) requires further comment. Both these recent papers describe experiments in which measurements are made of the amount of oxygen liberated on acidifying oxyhemoglobin. The addition of acid changes a certain portion of the oxyhemoglobin to methemoglobin (under certain conditions hematin may also be formed) and oxygen is liberated; the amount of oxygen thus set free corresponds to half that combined with the oxyhemoglobin before it was changed to methemoglobin. Since both investigations lead to the same result, although the experimental details differed, this relationship appears to be well established. *It does not follow, however, that the amount of oxygen bound in methemoglobin is one-half of that bound in oxyhemoglobin.* All that these experiments establish is the amount of gaseous oxygen consumed in the change from hemoglobin to methemoglobin; this is found to be one-half that bound in oxyhemoglobin. We have shown, however, that the amount of ferricyanide required to accomplish the same result is equivalent to one-quarter the amount of oxygen bound in oxyhemoglobin (1) and the same amount of reducing agent will cause the reverse process (Section I of this paper).

The apparent contradiction, then, is simply this: twice as much gaseous oxygen is used up in oxidizing hemoglobin to methemoglobin as would be predicted from the quantity of ferricyanide necessary for the same reaction or the amount of hyposulfite which will reverse it. There is nothing unusual in such a state of affairs; gaseous oxygen is a notoriously peculiar type of oxidizing agent and very different from substances like ferricyanide. Many examples are on record where more oxygen is consumed in a process of oxidation than corresponds to the equivalents of oxidation taking place, hydrogen peroxide or its salts being formed. Thus, the oxidation of phenols (21) and hydroquinones (22) is known to proceed in this manner. While no appreciable quantity of hydrogen peroxide probably persists as such in hemoglobin solutions, it is perfectly possible that an organic peroxide may be formed, or hydrogen peroxide is first formed and then is used up in reac-

tions with the protein. At any rate, from all the evidence available, it seems fairly certain that only half the gaseous oxygen absorbed in the oxidation of hemoglobin is involved in the formation of methemoglobin, the rest is used up in some other manner.

Oxyhemoglobin is essentially a metastable compound; it is a ferro compound holding 2 atoms of oxygen. The decomposition of solutions of this substance into methemoglobin and oxygen is very sensitive to catalysis and changes in hydrogen ion concentration. There are three possible paths for this reaction which are given below.



We hope to publish later some results concerning the various factors affecting the decomposition of hemoglobin solutions in the presence of oxygen. We may state here in advance that the evidence is very clear that in many cases of the formation of methemoglobin, equation (a) does not fit the observed facts and the choice lies between (b) and (c). There is every reason to believe this to be true in general and, therefore, strictly speaking, the problem is not the decomposition of oxyhemoglobin, but the oxidation of hemoglobin by oxygen or by oxyhemoglobin. Roaf and Smart's and Quagliariello's results, therefore, must be interpreted on this basis. We have suggested above a probable explanation in terms of equation (b); a similar scheme, if oxyhemoglobin is the oxidizing agent, is represented by (c).

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SUMMARY.

1. The electrometric titration of methemoglobin with sodium hyposulfite has been compared with the Van Slyke and Stadie methods of determining methemoglobin. The results afford additional evidence that one hydrogen equivalent is involved in the change from methemoglobin to hemoglobin.

2. The oxidation-reduction potentials of mixtures of hemoglobin and methemoglobin have been measured. The results are in general agreement with those predicted from the electrochemical equations and the values obtained by the titration method.

3. The reaction between potassium ferricyanide and hemoglobin solutions has been shown to be reversible, proceeding to a definite end-point dependent on the hydrogen ion concentration and the oxygen pressure above the solution. In alkaline solutions or at low oxygen pressures the reaction between equimolecular amounts is practically complete; in neutral solutions saturated with oxygen it is only about 80 per cent complete. The results are in accord with our electrochemical formulation of the hemoglobin-methemoglobin system and Hill's equation for the oxyhemoglobin-hemoglobin-oxygen system; such results afford a drastic test for an equation of this type.

4. It is shown that the behavior of hemoglobin, methemoglobin, and oxyhemoglobin is in accord with a complex ion formula previously suggested. The discrepancies between our electrometric titrations and the results of two recent investigations on the formation of methemoglobin by acids are discussed and a probable explanation of the apparent contradiction is given.

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A METHOD FOR DETERMINING METHEMOGLOBIN IN THE PRESENCE OF ITS CLEAVAGE PRODUCTS.

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Aside from the use of a spectrophotometer, the methemoglobin content of a solution is usually estimated indirectly by determining the total amount of pigmented protein and the reduced hemoglobin, and assuming the difference to represent methemoglobin. Thus, the procedure of Stadie (1) and of Van Slyke and Stadie¹ makes use of the cyanhemoglobin colorimetric method for determining so called total hemoglobin (*i.e.* the sum of reduced and methemoglobin) and the oxygen capacity for determining the reduced hemoglobin. This procedure for total hemoglobin is very satisfactory if no other colored substances are present, but is not applicable in the presence of the colored cleavage products of hemoglobin (hematin). This became evident to us in the course of some experiments with hemoglobin solutions which had undergone considerable decomposition; we compared the amount of sodium hyposulfite required in an electrometric titration (3) and the percentage of methemoglobin by the Van Slyke-Stadie method, and found the ratio to be very different from that obtained with pure hemoglobin. By adding varying amounts of hematin to solutions of hemoglobin it was further demonstrated that the presence of such cleavage products greatly affected the colorimetric determination of the total hemoglobin content. Spectrophotometric methods would probably not be affected in this manner, although it is possible that the presence of certain colored substances might make even the use of this instrument unreliable for the determination of methemoglobin. As we desired to determine methemoglobin in a variety of solutions and a spectropho-

¹ Van Slyke and Stadie (2), p. 32.

tometer was not available, we developed a simple procedure for determining total hemoglobin in the presence of its cleavage products and any other colored substance. This method may be found useful as an adjunct to the spectrophotometric procedure and, as it requires no special apparatus, may recommend itself to others who for one reason or another cannot employ an optical method.

The method depends on the fact that methemoglobin is quantitatively changed to reduced hemoglobin by certain powerful reducing agents (3), and the amount of reduced hemoglobin thus formed can be determined by the increase in oxygen capacity of the solution after treatment with the reducing agent. Three reducing agents were tried, sodium hyposulfite, sodium anthrahydroquinone- β -sulfonate, and disodium anthrahydroquinone-2, 6-disulfonate. All three substances rapidly and quantitatively reduce methemoglobin, and apparently would be equally suitable for the purpose at hand. It was found, however, that the use of sodium hyposulfite was quite out of the question as, on shaking the reduced hemoglobin with oxygen, methemoglobin was rapidly formed again. Apparently the oxidation products of sodium hyposulfite are powerful catalysts for the oxidation of hemoglobin to methemoglobin.

This adverse experience with sodium hyposulfite, as well as some experiments on the decomposition of hemoglobin solutions to be described in another paper, made it evident that it would be necessary to employ some reducing agent whose oxidation products would influence slightly, if at all, the rate of formation of methemoglobin by oxygen. By using disodium anthrahydroquinone-2, 6-disulfonate as the reducing agent and vigorously shaking the solution for only 15 seconds with oxygen, it was possible to obtain fairly satisfactory results. These are recorded in Table I; the detailed procedure need not be described, as eventually this method was superseded by the use of the β -monosulfonate in the manner outlined below. The hemoglobin solution employed was freshly laked corpuscular cream from horse blood, and the oxygen capacities were determined in the usual manner in the Van Slyke apparatus. An inspection of the table shows that the reduced hemoglobin content could be increased by the action of the reducing agent to almost the original value of the solution before treat-

ing with ferricyanide. The average of the results obtained (Experiments 3 to 7, Table I), however, is about 0.50 per cent too low in total hemoglobin as compared with the original material; this, in terms of percentage error, is about 3.3 per cent, and is much greater than the errors of the Van Slyke method for determining the oxygen capacity. Evidently some of the reduced hemoglobin

TABLE I.

Determination of Total Hemoglobin by Reduction with Disodium Anthrahydroquinone-2, 6-Disulfonate.

Experiment No.	Saturated $\text{K}_3\text{Fe}(\text{CN})_6$ added per cc.	Hb content after addition of $\text{K}_3\text{Fe}(\text{CN})_6$.	Anthrahydroquinone solution used.	Time with O_2 .	Hb content after reduction.	Remarks.
	cc.	gm. per 100 cc.	cc. per cc.	min.	gm. per 100 cc.	
1	None.		None.		13.66	Blank determination giving Hb content of pure solution.
2	"		"		13.72	
3	"		0.2	0.25	13.35	
4	"		0.2	0.50	13.34	
5	0.0075	6.84	{0.2	0.25	13.28	
			{0.2	0.25	13.23	
6	0.010	4.35	{0.2	0.25	13.33	
			{0.2	0.25	13.26	
7	0.015	0.49	{0.3	0.25	13.25	
			{0.3	0.25	13.00	
8	None.		0.2	0.75	13.50	Illustrating effect of prolonging time with O_2 .
9	"		0.2	1	13.15	
10	"		0.2	2	13.16	
11	"		0.2	90	11.42	

Hb content determined by oxygen capacity in the Van Slyke apparatus; correction for physically dissolved oxygen applied as described under "Calculation of results."

was reconverted to methemoglobin even in the very short time the solution was shaken with oxygen. This was further shown to be the case by carrying out the last four experiments recorded in the table, in which the time of contact with the oxygen was increased; the decrease in reduced hemoglobin content is very evident, particularly in the last experiment.

While the results presented in Table I show that fairly satisfactory determinations of total hemoglobin can be made by using disodium anthrahydroquinone-2, 6-disulfonate, we desired to make the method still more accurate. To this end we substituted sodium anthrahydroquinone- β -sulfonate for the disulfonate, since preliminary experiments seemed to indicate that this substance was somewhat less effective in catalyzing the formation of methemoglobin. Thus, in comparing the action of equal weights of the β -sulfonate and disulfonate on pure hemoglobin solution, it was found that the former produced only 5.7 per cent of methemoglobin, while the latter produced 7.0 per cent in a given time. The reduction potential of the β -sulfonate is even lower than that of the disulfonate (4), so that it is perfectly suitable from this point of view.

Experiments in which the β -sulfonate was used as a reducing agent showed that the determination of total hemoglobin with this reagent was very satisfactory. They are recorded in Table II; the exact experimental procedure which is recommended for use as an analytical method is given below. The methemoglobin content of the solution after the addition of the ferricyanide in Experiments 5 and 6 was estimated from our experiments on the ferricyanide reaction described in the preceding paper (3). Similarly the methemoglobin content after the addition of 0.5 equivalent of benzoquinone (Experiment 7), and after shaking with air for 1 hour in the presence of anthraquinone disulfonate (Experiment 8), could be approximately stated from work which is soon to be published. The exact amount of methemoglobin was of little importance in showing the validity of the analytical procedure, and the oxygen capacity of the solution was, therefore, not determined at this point. That the success of the procedure is in no way dependent on the reagent used for forming the methemoglobin, is demonstrated by these last two experiments.

An examination of the last column of Table II shows that the total hemoglobin content of the solution after considerable methemoglobin had been produced by various reagents agrees well with the blank determinations made with the pure hemoglobin solution (Experiments 1 and 2). The error is not more than 3 parts in 130, and in most cases is considerably less than this.

Procedure.

Preparation of Sodium Anthrahydroquinone- β -Sulfonate.—The commercial sodium anthraquinone- β -sulfonate is usually about 90 per cent pure and is satisfactory for the preparation of the anthrahydroquinone compound. This is accomplished by shaking a solution of the anthraquinone sulfonate with platinized asbestos and hydrogen, preferably under slight pressure. A solution of convenient strength is prepared by dissolving 1.5 gm. of the sodium anthraquinone- β -sulfonate in 100 cc. of a 0.025 M mixture of disodium and monosodium phosphates of pH about 6.4. This, together with a small amount of platinized asbestos, is placed in a small bottle provided with a hydrogen inlet entering below the surface of the

TABLE II.

Determination of Total Hemoglobin with the Aid of Sodium Anthrahydroquinone- β -Sulfonate.

Experiment No.	Reagent employed for producing methemoglobin.	Approximate amount of MHb formed.	Anthrahydroquinone solution used per 4 cc.	Hb content after reduction.
		gm. per 100 cc.	cc.	gm. per 100 cc.
1	None.	None.	None.	12.91
2	"	"	"	12.89
3	"	"	0.5	12.96
4	"	"	0.5	12.77
5	0.5 equivalent of $K_3Fe(CN)_6$.	4.7	0.6	12.87
6	0.7 " " "	7.1	0.9	12.59
7	0.5 " " benzoquinone.	3.7	0.7	12.65
8	1.0 " " anthraquinone-2, 6-disulfonate and 1 hr. with air.	4.0	0.6	12.94

The hemoglobin solution employed was freshly laked corpuscular cream from horse blood.

liquid, a gas outlet provided with a stop-cock, and a tube extending to the bottom of the vessel; this tube ends in a small inverted funnel provided with a Witt filter plate covered with cotton gauze. On the upper end is a piece of vacuum rubber tubing closed by a pinch-cock, and into which the tip of a very small burette (of a few cc. capacity)² could be fitted for removing the anthrahydroquinone solution. After saturating the solution with hydrogen, the outlet is closed and the bottle shaken for 1 to 2 hours, which completes the reduction. If desired, the amount of hydrogen absorbed can be measured by a suitable measuring apparatus, and the

² Conveniently made by sealing a glass stop-cock on the bottom of the usual graduated 2 or 5 cc. pipette (uniform bore).

progress of the reduction followed in this way. The resulting highly colored solution is 0.036 molar or 0.072 normal in respect to its reducing power, which may be determined by electrometric titration against potassium ferricyanide. It is very sensitive to air, and must be handled rapidly and protected against the action of oxygen by a layer of xylene in the burette from which it is measured.

Determination of Total Hemoglobin.—A carefully measured 4 cc. sample of the hemoglobin solution is freed from oxygen in a 250 cc. tonometer by evacuation and filling with nitrogen in the usual manner. The anthrahydroquinone solution is then forced from the bottle in which it was prepared, through the tube previously described, into the micro burette by the hydrogen pressure, a small amount of xylene being previously introduced into the burette. From 0.5 to 1.0 cc. of this anthrahydroquinone solution (the amount depending on the concentration of methemoglobin present) is then rapidly introduced into the nitrogen-filled tonometer, which is then evacuated. The tonometer is then given a few rotations to insure complete mixing, filled rapidly with oxygen (at atmospheric pressure), shaken vigorously for 5 to 10 seconds, and a measured sample withdrawn by means of an Ostwald-Folin pipette and at once introduced into the Van Slyke apparatus for determining oxygen capacity.

The determination of the oxygen combined in the sample is carried out exactly as described by Van Slyke and Neill (5). Even using the β -sulfonate, it is necessary to analyze the sample immediately after it has been shaken with oxygen, and to restrict the shaking with this gas to 5 to 10 seconds of vigorous agitation. Experiments showed that the time during which the reducing agent is in contact with the solution is of no significance and may be as long as 20 minutes. The only point at which especial pains must be taken to work rapidly is during the equilibration with oxygen, and subsequent removal of a sample to the Van Slyke apparatus.

Calculation of Results.—The calculation of the oxygen content of the sample from the difference in pressure is made according to the formula given by Van Slyke. Because of the fact that the hemoglobin is equilibrated with pure oxygen and not air, the correction for dissolved oxygen is different from that usually employed. We have taken the amount of oxygen *physically dissolved* in hemoglobin equilibrated with pure oxygen at 38° and 760 mm. (710 mm. partial pressure) as 1.89 cc. per cc., by extrapolation of data given by Van Slyke and Stadie.³ Using this value and Bohr's solubility data (α = cc. of gas at 0°, 760 mm., dissolved by 1 cc. of water), we have calculated the correction for the dissolved oxygen at different temperatures from the following formula:

$$(\text{Cc. of O}_2 \text{ per 100 cc.})^t = 1.89 \times \frac{\alpha^t}{0.022} \times \frac{760 - \text{aqueous tension}}{710}$$

³ Van Slyke and Stadie (2), p. 17. *

using $\alpha = 0.031$ at 15° , 0.029 at 20° , 0.027 at 25° , and 0.022 at 38° . It is convenient to express this in terms of percentage of hemoglobin, and the correction can then be directly subtracted from the uncorrected hemoglobin content calculated directly from the readings of the Van Slyke apparatus. It is very probable that later work may change the value of this correction for physically dissolved oxygen, and we consider the correction we have employed merely as a suitable approximation.

The corrected hemoglobin content of the sample must then be converted into percentage (gm. per 100 cc.) of total hemoglobin in the original solution. This is done by multiplying by the factor $\frac{1+m}{1}$, where m is the cc. of reducing agent employed per cc. of hemoglobin solution. In the experiments given in Tables I and II, since we also added varying amounts (n) of ferricyanide solution, etc., the factor $\frac{1+m+n}{1}$ was employed to reduce all the results to a comparable basis.

Knowing the total hemoglobin content of the solution, and having determined the oxygen capacity of the original solution and thus the reduced hemoglobin content (corrected, of course, for physically dissolved oxygen), the percentage of methemoglobin is obtained by difference. Or, if one prefers, the results may be expressed in terms of oxygen capacities before and after treatment with the reducing agent, and the change in oxygen capacity then converted to percentage of methemoglobin. This treatment of the data makes it evident that the correction for physically dissolved oxygen affects the value of the methemoglobin content but slightly; indeed, if no increase in volume attended the addition of the reducing agent, the correction would completely cancel. As it is, it amounts to mZ , where m is the cc. of reducing agent per cc., and Z the correction for physically dissolved oxygen expressed in percentage of hemoglobin; since m is 0.1 to 0.3 and Z not more than 2 per cent, the value of Z might be considerably in error without influencing very much the methemoglobin content.

Whichever of the methods of calculation is employed, it is apparent that what one is really measuring is the amount of material which by itself has no oxygen capacity, but which after treatment with a reducing agent combines with oxygen reversibly. As far as is known, *the only substance which has this very peculiar property is methemoglobin.*

A few experiments were carried out to see whether the presence of the oxidized form of the reagent employed (*i.e.* the anthraquinone sulfonate) influenced appreciably the amount of physically dissolved oxygen. The results given in Table III show that the correction we have employed is applicable to dilute solutions and those containing the organic and inorganic substances used in our work; the oxygen capacities were corrected as outlined above, the solutions being equilibrated with pure oxygen, and the amount of hemoglobin in the original solution calculated by use of the factor $\frac{1+w}{1}$, where w is the cc. of diluent per cc. of hemoglobin.

TABLE III.
Hemoglobin Content of Solutions Diluted with Various Diluents.

Diluent.	Percentage Hb.		
	Uncorrected.	Corrected for physically dissolved O ₂ .	In original solution (corrected).
None.....	13.93	12.12	12.12
	13.93	12.04	12.04
	13.82	11.98	11.98
30 per cent H ₂ O.....	11.02	9.30	12.09
30 " " reducing agent.....	11.00	9.26	12.03
100 " " H ₂ O.....	7.78	6.03	12.06
4.2 " " 2.5 M phosphate buffer.....	13.28	11.52	12.00

SUMMARY.

A method for determining methemoglobin in the presence of its cleavage products or other colored material has been developed. It consists in measuring the increase in oxygen capacity, resulting from the action of a powerful reducing agent which quantitatively converts the methemoglobin to hemoglobin. Sodium anthrahydroquinone- β -sulfonate is a satisfactory reducing agent for this purpose, although the oxygen capacity after reduction must be determined rapidly, and the equilibration with pure oxygen must be of very short duration. Disodium anthrahydroquinone-2, 6-disulfonate is a somewhat less satisfactory reagent, while sodium hyposulfite cannot be used at all because its oxidation products catalyze so greatly the reformation of methemoglobin.

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THE ENZYMATIC SYNTHESIS OF PROTEIN. II.

THE EFFECT OF TEMPERATURE ON THE SYNTHESIZING ACTION OF PEPSIN.

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In a solution of the products of the hydrolysis of protein it is theoretically possible to bring about the reverse reaction, *i.e.* synthesis, in two ways: by concentrating the solution, and by raising the temperature. The theoretical considerations from which the first of these conclusions was deduced have been discussed in a previous paper (1). It is sufficient to recapitulate here, that the first method is predictable from an appropriate statement of the mass law. The experimental confirmation of the prediction was described by the authors (1). The second method is predictable from certain thermodynamical considerations of reversible reactions pointed out by Moore (2). He deduced the equilibrium equation $P_a = K P_b^n$, where P_a and P_b are respectively the osmotic pressures of the substrate and its product, and K is a constant. K is a symbol for the expression $P_0 e^{\frac{C}{RT}}$, where P and e are constants, R is the gas constant, C is the chemical energy involved in the breakdown of 1 gram molecule of A into n gram molecules of B , and T is the absolute temperature.

As Moore points out, it is obvious, from an inspection of the functions combined in the value of K , that an increase of temperature should have the reverse effect of an increase in the value of C .

A large value for C obtains always in reactions where reversibility is difficult of attainment. It follows that, especially in those reactions where C has neither a very high positive nor a very high negative value, an increase of temperature should

facilitate reversibility. In such cases, at high temperatures, synthesis may be possible where, at low temperatures, hydrolysis was in progress.

The value of C for the hydrolysis of proteins is practically zero (3). Protein hydrolysates present, therefore, almost an ideal system for testing the effect of temperature on reversibility.

EXPERIMENTAL.

A 6 per cent solution of egg albumin (Merck) was digested with 0.1 per cent pepsin (Merck) at pH 1.7 for 7 days. At the end of this period it gave no precipitate with either trichloroacetic or copper acetate, indicating that protein and primary proteose were absent. It was filtered, adjusted to pH 4.0, and without increasing the concentration, or adding any more enzyme, a portion was heated to 65°C. In 10 minutes a precipitate appeared, and in $\frac{1}{2}$ hour a large amount was obtained. This was filtered off, thoroughly washed, and dissolved in 0.025 N NaOH. A biuret test was carried out, and a typical protein color was obtained, while the color given by the digest maintained at 20°C. was the rose shade of proteose and peptones. Another smaller amount of this precipitate was dissolved in 0.025 N HCl, and, on the addition of trichloroacetic acid, a characteristic precipitate of plastein or native protein appeared. The solution at pH 4.0, maintained at 24°C., showed no precipitate, and remained perfectly clear over a period of 8 hours. Boiling the digest and then maintaining at 65°C. for 8 hours brought no increased precipitation over the faint coagulation produced on boiling. Some of the original solution was adjusted to pH 1.7, instead of to pH 4.0 (introducing the same dilution as in the adjustment to pH 4.0), and was then maintained at 64°C. for 6 hours. Only a slight precipitate was obtained, unlike plastein in appearance, and too slight to be filtered. This precipitate may have been protein, because a slight amount of synthesis is possible at pH 1.7.

We have shown (1) that the insoluble material formed under such conditions is a product synthesized from the components of the digest, and that it is of the complexity of protein. We feel justified, therefore, in referring to this product in future, simply as protein.

The effect of temperature is illustrated in another type of experiment.

0.5 gm. of pepsin (Merck) was dissolved in 0.1 N HCl in each of eight flasks, brought to the temperature indicated in Table I, and maintained there for $\frac{1}{2}$ hour. 10 cc. of a concentrated solution of the products of peptic hydrolysis, at the same temperature and at pH 4.0, were added, thoroughly mixed, and set away with chloroform at this temperature for 24 hours. At

the end of this period the flasks were removed, their contents neutralized to stop any further peptic action, and diluted to 100 cc.

The resulting mixtures were then analyzed for the insoluble protein by estimating the total nitrogen before and after filtration.

Percent

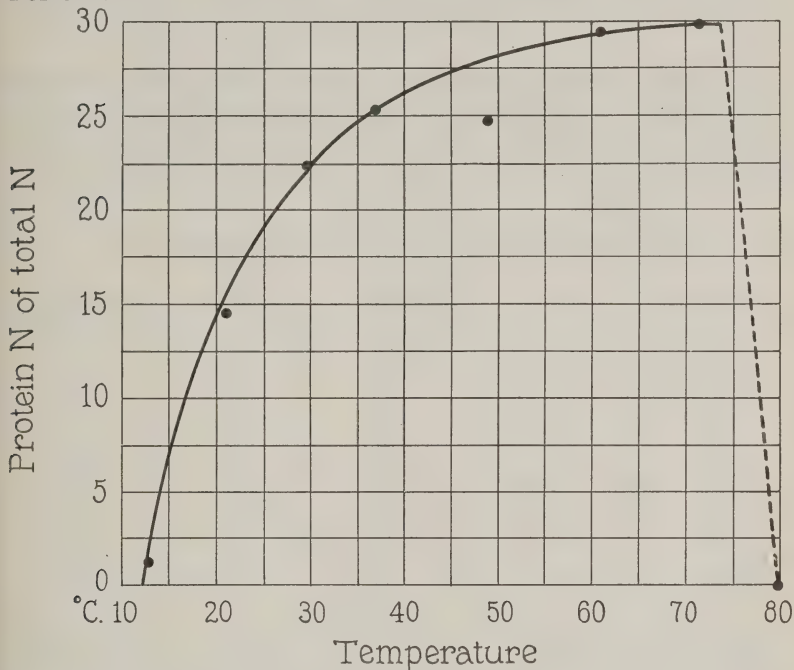


FIG. 1. Effect of temperature on the amount of protein formed.

As will be shown in a later paper, with the possible exception of the lowest temperature, these represent, in all probability, close approximations to equilibrium amounts. The effect of increase of temperature, then, is to move the equilibrium point more and more over to the protein side, *i.e.* to increase the possibility of reversion (Fig. 1).

There seems to be no necessity for postulating, as Robertson (4) does, two enzymes or two components of one enzyme, one functioning in hydrolysis and the other in synthesis. Assuming only the classical function of a catalyst, a shift in equilibrium is accounted for on thermodynamic grounds, when the only influence ascribed to the enzyme is the acceleration of the attain-

ment of the point of equilibrium, wherever that point may be defined by the heat, chemical energy, and osmotic pressures of the reacting components.

Inseparability of the Hydrolyzing and Synthesizing Components of Pepsin.

Specially designed experiments showed that the processes of boiling and of addition of alkali, which destroyed the hydrolyzing protease pepsin, also resulted in the destruction of the synthesizing power of the commercial pepsin preparation.

TABLE I.
Effect of Temperature on Peptic Synthesis.

Temperature.	Total N.	N in filtrate.	Protein N.	Protein N of total N.
°C.	mg.	mg.	mg.	per cent
13	818	809	11	1.5
21	811	693	118	14.5
30	828	642	186	22.4
37	397	297	100	25.3
49	830	824	206	24.9
61	806	568	238	29.5
72	823	576	247	30.0
80	823	823	0	0

Four flasks, Nos. 1, 2, 3, and 4, were set up. In Flask 1, 1.00 gm. of pepsin (Merck) was dissolved in 2 cc. of 0.1 N HCl, giving clear solution, and was set away at 37°C. for 1 hour. The solution remained clear. 20 cc. of concentrated peptic digest at pH 4.0 were pipetted in, and thoroughly mixed. Chloroform was added and the flask was tightly stoppered and set away at 37°C. for 24 hours. In 10 minutes a precipitate appeared in the clear solution, and in 4 hours the formerly quite fluid digest was transformed into a solid opaque jelly. In Flask 2, 1.00 gm. of pepsin (Merck) was dissolved in 2 cc. of 0.1 N HCl and set away at 37°C. for 1 hour. The flask was then placed for 15 minutes in boiling water and for $\frac{1}{2}$ minute on a hot-plate. The solution was coagulated. It was then cooled and thoroughly mixed with 20 cc. of concentrated digest. Quite unexpectedly the coagulum dissolved, leaving only a slight turbidity which did not increase on standing 24 hours at 37°C. In Flask 3, 1.00 gm. of pepsin was dissolved in 1 cc. of 1.0 N NaOH, alkali of this concentration being required to render the solution alkaline to litmus. A precipitate appeared immediately. It was set away at 37°C. for 1 hour and then neutralized with 1.5 N HCl so that the solution became acid to litmus. 20 cc. of the concentrated peptic digest were added. After

thorough stirring, this precipitate also dissolved, leaving a slight turbidity which did not increase during the 24 hours standing at 37°C. Flask 4 was identical in contents with Flask 3.

At the end of 24 hours, the contents of Flasks 1, 2, and 3 were neutralized, diluted to 250 cc., and analyzed for protein. The contents of Flask 4 were used for the estimation of the pH, which was found to be 4.0. The results of the analysis are given in Table II.

457 mg. of protein nitrogen were formed in Flask 1, none in Nos. 2 or 3. The turbidity of the coagulated enzyme yielded little or no nitrogen, a surprising result, which has been confirmed on a number of occasions. The results are unequivocal; boiling and alkali destroy the synthesizing power of commercial pepsin. The statement of Bayliss (5) that alkali, which destroys the

TABLE II.

Effect of Boiling and Treatment with Alkali on the Synthesizing Power of Pepsin.

	Flasks.		
	1 Normal pepsin.	2 Boiled pepsin.	3 Alkali- treated pepsin.
	mg.	mg.	mg.
Total N before filtration.....	1,802	1,752	1,789
“ “ after “	1,345	1,752	1,785
Protein N.....	457	0	4(?)

hydrolytic activity of pepsin, does not remove the precipitating power, is not applicable to our experiments. Bayliss, in discussing Robertson's results, suggests that the synthesis (precipitation), described by him, was probably due to the particular sample of pepsin employed. It seems possible that the synthesis obtained by Robertson is similar to that known as plastein formation, and it may therefore be worth pointing out that protein (plastein) has been obtained by Danilewski and other Russian workers with stomach extract. Robertson employed Grubler's pepsin, and we have employed Eimer and Amend's pepsin and two batches of Merck's.

Further, to remove another objection by Bayliss, the acid peptic hydrolysates utilized by us were in every case filtered be-

fore concentration, and this in no way prevented the formation of protein in our digests of egg albumin.

So far no means of separating the hydrolyzing and synthesizing components had presented itself. Treatment which destroys one also destroys the other. But in view of the statement found in the literature (Bayliss (5) and Robertson (4)) that the hydrolyzing component of pepsin is destroyed at 65°C., a method seemed to be available. At 72°C. the maximum yield of protein was obtained, and if heating at 65°C. under the conditions of protein formation destroys the hydrolyzing power of pepsin, the dissimilarity of the synthesizing and hydrolyzing components is proven.

TABLE III.

Effect of Temperature on the Hydrolyzing Component of Pepsin.

Flask No.	Time.	Free amino nitrogen.
	hrs.	mg.
	0	0.62
1	27	2.97
2	27	2.47
3	27	0.99

In each of three flasks, Nos. 1, 2, and 3, 0.4 gm. of Merck's pepsin was dissolved in 1 cc. of HCl, the resulting pH being 3.3. No. 1 was maintained at 60°C. for $\frac{1}{2}$ hour; No. 2 at 72°C. for $\frac{1}{2}$ hour; and No. 3 at 80°C. for $\frac{1}{2}$ hour. At the end of this period Nos. 1 and 2 were quite clear, and No. 3 was coagulated. 50 cc. of 3 per cent albumin at pH 1.7 were added to each, and the flasks tightly stoppered and set away with chloroform at 37°C. Free amino nitrogen estimations were carried out on 5 cc. of the solution immediately, and 27 hours later. The results are given in Table III.

Flasks 1 and 2, after 27 hours, gave turbidities only, when trichloroacetic acid was added, while No. 3 gave a voluminous, flocculent precipitate. The slight increase in the amino nitrogen in No. 3 was probably due to the hydrolyzing effect of the acid.

The results of this experiment indicate that temperatures of 60°C. and 72°C., maintained for $\frac{1}{2}$ hour, do not destroy the hydrolyzing power of pepsin. Robertson, and also Bayliss, may have overlooked the possibility of the protective influence of concentrated solutions of the products of hydrolysis on the enzyme.

Our previous experiment proved that the processes of boiling and addition of alkali destroy not only the hydrolyzing, but also the synthesizing properties of our pepsin preparation. The last experiment proves that maintenance at a temperature of 72°C. which gives a maximum synthesis does not, as might have been supposed, destroy the hydrolyzing properties, and the only conclusion possible is that the hydrolyzing and synthesizing components are inseparable and are in all probability identical. The higher yield of protein at 72°C. is predictable without postulating any influence of temperature upon the condition of the catalyst. The action of the latter is to accelerate the attainment of equilibrium wherever that point is defined by the temperature and the concentration, whether on the side of hydrolysis or of synthesis.

SUMMARY.

1. In concentrated peptic hydrolysates of protein, at pH 4.0, in the presence of pepsin, increasing amounts of protein are formed with increasing temperature, up to the point of destruction of the enzyme.
2. This result is shown to be predictable from certain thermodynamical considerations of reversible reactions.
3. The synthesizing and hydrolyzing components of commercial pepsin were found to be inseparable.

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THE OXYGEN AND CARBON DIOXIDE CONTENTS OF THE ARTERIAL AND MIXED VENOUS BLOOD IN NORMAL INTACT DOGS.

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In the course of some experiments in another connection we had occasion to study the oxygen and carbon dioxide contents of the arterial and of the mixed venous blood in normal intact dogs. Because of the absence of such data in the literature and because of the increasing use of dogs in the study of respiratory and circulatory problems we are reporting these observations.

The desirability of obtaining samples of mixed venous blood in intact dogs without the use of general anesthetics is obvious. In order to estimate the oxygen and carbon dioxide in mixed venous blood in intact dogs two methods are in use: (1) A tonometer method in which alveolar air and pulmonary venous blood are allowed to come into equilibrium; the air is then analyzed. The objections to this method are quite clear. (2) A method of direct puncture of the right auricle or ventricle in order to obtain mixed venous blood. Attempts to use this method showed that it was quite unreliable, because it was impossible to judge with certainty from which heart chamber the blood sample was obtained, in spite of the use of x-ray photographs to show the size and location of the heart. Dissection of fresh dog cadavers after heart punctures with the needles left *in situ* confirmed the liability to error involved in this method. For this reason we developed the method which is now described.¹

We devised a cannula² which can be inserted into the right ventricle through the right external jugular vein. Through this,

¹ A preliminary report of this method appeared in the Proceedings of the Society for Experimental Biology and Medicine (Stewart, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 378).

² The instrument was made by Tiemann and Co., New York.

samples of mixed venous blood are withdrawn from the right heart. The cannula (Fig. 1) is a hollow metal tube 2.5 mm. in diameter



FIG. 1. Trocar and cannula for withdrawing blood from the right ventricle in dogs. *a*, obturator partly withdrawn from the cannula; *b*, stop-cock; *c*, outlet for withdrawing blood samples; *d*, hollow tube; *e* shows the obturator in position before inserting cannula into the right ventricle.

and 30 cm. long, having an obturator which is bullet shaped closing its distal end. When the obturator is withdrawn the proximal opening is closed by a small stop-cock. Below the stop-cock is an outlet to which a piece of rubber tubing is attached. Before use the air in the cannula is replaced by sterile alboline.

The dog lies quietly on the operating table. The operation is carried out aseptically. The skin and subcutaneous tissues along the line of incision are anesthetized with 2 per cent novocaine. The right external jugular vein can then be exposed by blunt dissection without discomfort to the dog and without any emotional disturbance. The distal end of the vein is ligated. A small opening is made into the vein below the ligature and the cannula inserted and tied in place. The cannula with the obturator in place is pushed into the innominate vein, superior vena cava, and then into the right heart. At first the operations were carried out under the guidance of the fluoroscope, but this was soon found to be unnecessary, because by turning the dog slightly on his left side the line along the external jugular vein to the heart becomes practically straight and the cannula enters the heart easily. When in place the cannula can be felt to move with each cardiac systole. When the obturator is withdrawn and the stop-cock closed, blood flows from the outlet. The blood is collected by placing the tube under alboline or, more quickly, by using an air-free syringe attached to the tube. Failure to obtain a sample usually results when the opening of the cannula presses too closely against the heart wall or when

one of the leaflets of the tricuspid valve is drawn against the opening on the application of suction by the piston of the

syringe. Shifting the position of the cannula a few millimeters will overcome this difficulty.

After a satisfactory sample has been obtained, the obturator is returned, the cannula removed, and the vein ligated below the point of entry. The skin is closed with a silk suture and protected with a dry dressing. The procedure requires less than 15 to 20 minutes. By inserting the cannula as high in the neck as possible the first time, the operation can be repeated many times, each time inserting the cannula below the point of the previous ligation. Occasionally at the first operation the cannula can be inserted through a small vein entering the external jugular vein, thus preserving the external jugular vein intact for subsequent operations. The vein remains patent below, but in time is obliterated above the point of ligation. When it is no longer possible to use the external jugular vein the internal jugular vein can be used in a similar manner. The left external jugular vein is more difficult to use because of the angle at which the vein enters the innominate vein.

Samples of blood from the left heart may be obtained from the right carotid artery at the time the external jugular vein is exposed or may be obtained more easily from a femoral artery. The femoral artery in the dog is quite superficial and an arterial puncture is easily and quickly done without discomfort and with safety if pressure is applied over the site of the puncture for a few minutes after withdrawing the needle. In these studies the sample of mixed venous blood was first obtained and immediately afterward the arterial sample was withdrawn from the femoral artery. The time interval between the taking of the two samples was never greater than 2 to 3 minutes.

The oxygen and carbon dioxide contents of the arterial and mixed venous blood were estimated in 19 normal mongrel dogs. The analyses for the oxygen and carbon dioxide contents were made by the method of Van Slyke and Neill, using the Van Slyke manometric apparatus.³ Duplicate analyses were made of a large number of the samples and the variations in these were not greater than 0.10 volume per cent for oxygen and 0.25 volume per cent for carbon dioxide. Although the number of dogs used in these studies is small, we can nevertheless draw conclusions on

³ Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.

TABLE I.

Dog No.	Date.	Weight. kg.	Oxygen.		Oxygen consumption. vol. per cent	Carbon dioxide.		Difference in carbon dioxide. vol. per cent	O ₂ capacity.		Oxygen saturation.		Coefficient of utilization.
			Arterial blood.	Mixed venous blood.		Arterial blood.	Mixed venous blood.		Arterial blood.	Mixed venous blood.	Arterial blood.	Mixed venous blood.	
	1924		vol. per cent	vol. per cent		vol. per cent	vol. per cent		vol. per cent	vol. per cent	per cent	per cent	per cent
127	Jan. 24	13.25	18.01	15.16	2.85	47.96	56.12	8.16	20.90	20.93	86.2	72.2	14.0
89	Mar. 20	9.15	19.83						21.58		91.9		
	" 21	9.15	18.76	16.01	2.75	45.78	50.66	4.88	20.88		89.8	76.5	13.3
130	May 22	22.95	21.99	16.35	5.64	44.44	50.80	5.36	24.30		90.5	67.2	23.3
132	Mar. 12	10.75	19.28	14.26	5.02	40.43	42.36	1.93	21.28	20.97	90.6	68.0	22.6
136	May 27	16.60	11.42	8.57	2.85	47.85	49.88	2.03	12.52		91.2	68.4	22.8
122	Jan. 16	20.97	21.48			34.04			23.22		92.5		
	Feb. 5	22.08	20.95	17.44	3.51	40.87	44.39	3.52	22.86	22.97	91.6	75.9	15.7
135	Apr. 8	18.80	22.02	18.45	3.57	42.50	47.24	4.74	23.89		92.2	77.2	15.0
137	" 17	16.45	20.59	17.38	3.21	48.93	53.16	4.23	22.30		92.3	77.9	14.4
101	Mar. 5	16.95	16.90	14.09	2.81	41.67	47.49	5.82	18.25		92.6	77.2	15.4
90	Feb. 27	11.10	18.31	15.69	2.62	42.86	46.24	3.38	19.70		92.9	79.4	13.5
134	Apr. 15	18.00	19.33	15.42	3.91	38.46	42.04	3.58	20.90		92.5	73.8	18.7
142	June 10	16.25	20.22	17.66	2.56	43.83	46.12	2.29	21.73		93.1	81.2	12.9
143	" 11	18.00	18.23	16.11	2.12	43.50	45.86	2.36	19.50		93.4	82.6	10.8
131	Feb. 19	10.67	21.42	16.16	4.76	42.10	43.35	1.25	22.81		93.9	73.0	20.9
133	Mar. 25	12.00	20.96	17.65	3.31	45.89	49.63	3.74	22.26		94.2	79.2	15.0
125	Jan. 10	13.10	20.04			39.66			21.29		94.1		
	" 11	13.10	17.90			42.93			18.99		94.3		
124	Feb. 21	19.10	19.05	14.80	4.25	44.02	44.51	0.49	20.14		94.6	73.9	20.7
129	" 14	13.12	15.70	13.03	2.67	43.77	45.58	1.81	16.58		94.7	78.5	16.2
88	June 5	7.90	17.00	13.69	3.31	43.74	46.36	2.62	17.68		96.1	77.4	18.7

the normal arterial and mixed venous oxygen saturations of normal dogs. If we disregard the observations made on Dogs 88 and 127 (Table I), because, as will be seen, these are the exceptional dogs, we have 20 observations made on 17 dogs, the arterial

TABLE II.

Grouping of the Arterial Bloods by Percentage of Oxygen Saturation.

Percentage of saturation.....	86-87	89.8-91	91-92	92-93	93-94	94-95	96-97
Dog. No.....	127	89 130 132	89 136 122	122 135 137 101 90 134	143 131 142	133 125 125 124 129	88
Total No.....	1	3	3	6	3	5	1
		3 = 15 per cent.	3 = 15 per cent.	14 = 70 per cent.			
		20 = 100 per cent.					

TABLE III.

Grouping of Mixed Venous Bloods by Percentage of Oxygen Saturation.

Percentage of saturation.....	67-68	72-73	73-74	75-76	76-77	77-78	78-79	79-80	81-82	82-83
Dog No.....	130 132 136	127 131	134 124	122	89	135 137 101 88	129	90 133	142	143
Total No.....	3	2	2	1	1	4	1	2	1	1
	3 = 17 per cent.	13 = 72 per cent.							2 = 11 per cent.	
	18 = 100 per cent.									

oxygen saturation ranging from 89.8 to 95 per cent (Table II). The range in 14 of these observations, or 70 per cent, lies between 92 to 95 per cent; these figures we can accept as the arterial oxygen saturation which is normal for the greatest number of intact

resting dogs. Of these 14 observations the greatest number is seen to lie in the range from 92 to 93 per cent. Of the 20 observations 3, or 15 per cent, are in the 89.8 to 91 per cent range of saturation and 3, or 15 per cent, in the range of 91 to 92 per cent. From these figures we see that in most dogs normal oxygen saturation of the arterial blood is consistently slightly less than in human arterial blood (95 per cent). That the normal arterial oxygen saturation of dogs under resting conditions remains constant within small limits is shown quite distinctly in Dogs 89, 122, and 125, in which there are two observations on consecutive days or after a longer interval of time.

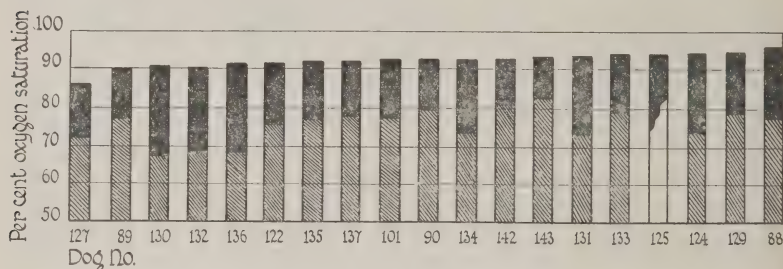


FIG. 2. The height of a column represents the percentage of oxygen saturation of the arterial blood, and the height of the area ruled with diagonal lines, the percentage of oxygen saturation of the mixed venous blood. The solid area corresponds to the coefficient of utilization of the oxygen-carrying power of the blood. A sample of the mixed venous blood was not obtained from Dog 125.

There are 18 observations on the oxygen saturation of the mixed venous blood in 18 dogs. In 13, or 72 per cent of these observations, the saturation of the mixed venous blood was within the range of 72 to 80 per cent (Table III); in 3, or 17 per cent, between 67 and 68 per cent; and in 2, or 11 per cent, between 81 and 83 per cent. In computing the saturation of the mixed venous blood the oxygen capacity of the arterial blood was used in most instances. That this is justified is shown in Dogs 127, 132, and 122 in which oxygen capacity of the mixed venous blood was also determined. The two are seen to agree closely, showing that the concentration of the red blood corpuscles in the arterial and the mixed venous blood is the same. The arterial and mixed venous oxygen percentage saturations are shown graphically in Fig. 2.

The usual range of the carbon dioxide content of the arterial blood was 40 to 45 volumes per cent, and of the mixed venous blood 45 to 50 volumes per cent.

SUMMARY.

1. A method is described for obtaining samples of mixed venous blood in intact dogs.
2. The normal arterial oxygen saturation of intact resting dogs ranges from 92 to 95 per cent, with the greatest number lying between 92 and 93 per cent.
3. The normal oxygen saturation of the mixed venous blood under these same conditions, and obtained by the method described, is 72 to 80 per cent.
4. The arterial saturation for any one dog under these conditions appears to be constant over long periods of time.
5. There is no difference in the concentration of the red blood corpuscles in the right ventricular and the left ventricular blood.

THE LIBERATION OF AMMONIA IN TRYPTIC DIGESTION.*

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Among the products of the complete hydrolysis of proteins, as it may be effected by boiling with mineral acids, there appears invariably (or almost invariably) a certain amount of ammonia. The proportion of the total nitrogen assuming this form—the so called “amide nitrogen”—has been determined with accuracy for a great variety of proteins; and in a limited number of cases the rate at which the ammonia is liberated during the progress of hydrolysis has also been ascertained (1, 2).

That the digestion of protein with pancreatic extracts leads likewise to the production of ammonia was discovered by Hirschler (3) as long ago as 1886; and 2 years later the work of Stadelmann (4) placed beyond doubt the enzymatic nature of the phenomenon. In 1902 Mochizuki (5), as the outcome of a single set of experiments with serum albumin, reached the conclusion that trypsin, if permitted to act for a sufficiently long period (20 to 74 days), is capable of liberating from protein material practically its entire quota of amide nitrogen. The observations of Henriques and Gjaldbæk (6), incidental to their extensive study of different proteolytic agents, hardly supported this conclusion; for they showed that of six proteins, subjected through periods of from 40 to 73 days to the repeated action of trypsin, none yielded more than one-half of the ammonia that could be obtained by boiling with acid. Andersen (7) was unable to bring about a complete hydrolysis of amide groups even by the successive use of pepsin, trypsin, and erepsin. Andersen and Roed-

* The experimental data are taken from a thesis presented by Ralph G. Smith in partial fulfilment of the requirements for the degree of Master of Arts at the University of Toronto.

Müller (8), writing in 1915, believed that the liberation of ammonia from protein by the digestive enzymes takes place in fact so slowly that its production in this manner within the alimentary canal is hardly possible. Opinion having thus made a complete revolution, we find the cycle apparently recommenced in 1922 by Fränkel and Jellinek (9), who announce as something "entirely new" their discovery of notable quantities of ammonia in a 60 day pancreatic digest of casein. It is obvious that our knowledge of the extent to which ammonia may be liberated in tryptic digestion is anything but precise. With respect to the rate of its appearance we possess practically no information whatever.

In an effort to throw some additional light upon these points the present writers performed during the spring and summer of 1922 a series of experiments, which were reported to the American Society of Biological Chemists in the December following (10). We intended originally, before publishing any detailed account of these experiments, to pursue our study of the problem a little further. Unfortunately the opportunity to do so has not yet arisen; and in the meantime there has appeared a paper by Luck (11) which deals with certain aspects of the same general topic. We feel, therefore, that we should not further delay the publication of the results already obtained.

Our experiments were carried out upon three proteins—casein, gliadin, and Witte peptone. Of these the first two, casein and gliadin, were selected as examples of proteins containing respectively a medium and a high proportion of amide nitrogen; while Witte peptone served to test the effect of trypsin upon a protein already partially hydrolyzed by pepsin. The casein was prepared from a commercial sample by dissolving it in dilute sodium hydroxide and precipitating with acetic acid, this process of purification being repeated twice. The gliadin was obtained from wheat gluten by the method of Osborne and Harris (12). The peptone was the commercial product in its original state.

Experiment 1. Digestion of Casein.—50 gm. of air-dry casein were dissolved in 500 cc. of water containing the equivalent of 300 cc. of 0.1 N sodium hydroxide. To this solution there was added gradually, with constant stirring, so much normal hydrochloric acid that the mixture just failed to

give a pink colour with phenolphthalein. The pH, determined colorimetrically with phenol red, was then approximately 8.0. The solution was filtered and treated with sufficient tricresol to give a concentration of 0.5 per cent. Two portions, each of 250 cc., were then transferred to tightly stoppered weighed bottles, and the weight of casein solution taken was ascertained by reweighing to the nearest centigram. Into a third bottle were put 250 cc. of water, containing 0.5 per cent of tricresol and sufficient sodium carbonate to make the reaction identical with that of the casein solution. All three bottles were warmed to 38°C. and set in a thermostat adjusted to maintain that temperature. To the water and to one of the casein solutions there was then added, at an instant carefully noted, 1 gm. of an active trypsin powder.¹ By vigorous shaking the ferment was brought as rapidly and as completely as possible into solution, and the bottles were returned at once to the thermostat. The latter now held the three following parallel preparations, each alike in regard to reaction, and each protected by tricresol from bacterial action: (1) a mixture of casein and trypsin—the “protein digest”—which constituted, of course, the main experiment; (2) an aqueous solution of trypsin—the “enzyme control”—which would indicate what quantities, if any, of ammonia or free amino groups might arise during digestion from the enzyme itself; and (3) a solution of casein free from ferment—the “protein control”—serving to test the possible hydrolyzing effect upon the substrate of the mere alkalinity of the digest.

From each of the three bottles samples were withdrawn 1 hour, 24 hours, 5 days, 10 days, and finally 88 days after the addition of the trypsin.

The sampling of the two casein solutions was carried out in the following way. Two stoppered 25 cc. volumetric flasks, of which the first contained 4 drops of 20 per cent hydrochloric acid, were weighed to the nearest centigram. Into the first were pipetted at the proper moment 20 cc. of the casein digest, any further hydrolysis of which was inhibited by instantly mixing it with the acid. Into the second were measured 20 cc. of the casein control. Each flask was immediately restoppered, cooled to room temperature, and weighed again. The difference gave, of course, the weight of casein solution taken in each instance, the weight of trypsin in the digest being practically negligible. Each flask was thereafter filled to the mark with water, and determinations of ammonia and of amino nitrogen were carried out upon convenient aliquots of the total volume.² The treatment of the digest with acid had the slight disadvantage of producing a precipitate; but this was of so fine a character that it remained suspended for a long time after shaking, and therefore did not seriously interfere with accurate measurement. The results of the analyses were calculated to the total

¹ The trypsin used bore the label of a well known distributing house. It was almost entirely soluble in faintly alkaline water.

² In the later stages of the digestion, when tyrosine had crystallized out, we used 50 cc. flasks, diluted the sample after weighing to about 45 cc., warmed till the crystals passed into solution, cooled, made to the mark, and took for analysis volumes twice as large as before.

quantity of casein solution undergoing digestion, of which not only the volume (250 cc.) but also the weight was known.

The plan of sampling the casein by weight was followed in order to escape the inaccuracy that would have been involved in measuring the warm liquid by volume. In the case of the enzyme control this precaution was neglected. Samples for the determination of ammonia and amino nitrogen were in this case measured directly from the trypsin solution by pipettes, and the results were calculated to the total volume of 250 cc. The quantities of nitrogen to be measured were here so small in relation to the casein nitrogen that the error caused by variations in temperature could be safely neglected. The total nitrogen of the enzyme solution was determined in a portion taken immediately after the commencement of digestion. It amounted to 0.136 gm.

While the digestion was proceeding, the unused residue of the original casein solution was analyzed for total nitrogen and ammonia and free amino nitrogen. The analyses were carried out upon weighed samples delivered from a small weight burette. The results enabled us to calculate the total casein nitrogen of the digest and its control, as well as the free amino nitrogen of the unaltered protein and possible traces of ammonia preexisting in its solutions. From the nitrogen determination and the known nitrogen content of pure casein (15.65 per cent) it was estimated that the solution contained 6.7 per cent of the dry protein.

The analytical methods used were Kjeldahl's for total nitrogen, Van Slyke's micro method for amino nitrogen (13), and the aeration method, with the technique and apparatus of Van Slyke and Cullen (14), for ammonia.

Experiment 2. Digestion of Gliadin.—For the experiment with gliadin we had available only 15 gm. of material. This was dissolved in 150 cc. of 0.1 N sodium hydroxide, and the solution was adjusted to a pH of about 8.0 by treatment with successive small portions of normal hydrochloric acid, the precipitate forming upon each addition being brought again into solution by warming and vigorous stirring. Two portions of 70 cc. each were used as protein digest and protein control, the former being treated with 0.28 gm. of trypsin. The volume of digest being so small, only three observations were possible. These were made at the 1 hour, 24 hour, and 5 day periods. No separate enzyme control was run, the results with the enzyme control of the casein experiment being taken as equally applicable to the present one. In all remaining respects the two experiments were carried through in identical fashion; and in order to make the results the more readily comparable the gliadin data were calculated to an assumed digest volume of 250 cc. Taking the nitrogen content of gliadin as 17.66 per cent, its concentration in the digest was found to be 8.2 per cent.

Experiment 3. Digestion of Witte Peptone.—50 gm. of "peptone" were dissolved in 500 cc. of warm water, and the reaction was adjusted by addition of the necessary small quantity of sodium hydroxide. The filtered solution was then treated exactly as the casein solution of the first experi-

ment except that the final samples were taken at the 68th day. Once again a separate enzyme control was dispensed with. The substrate concentra-

TABLE I.
Gross Results and Controls.

	Protein digest.				Protein control.				Enzyme control.	
	Amount of N as		Per cent of total N as		Amount of N as		Per cent of total N as		Amount of N as	
	NH ₂ ⁻	NH ₃	NH ₂ ⁻	NH ₃	NH ₂ ⁻	NH ₃	NH ₂ ⁻	NH ₃	NH ₂ ⁻	NH ₃
A. Casein. Total N of digest 2.786, of substrate 2.65 gm.										
	mg.	mg.			mg.	mg.			mg.	mg.
Original solution...					152	0.51	5.73	0.02		
After 1 hr.....	1,011	8.84	36.3	0.32	152	0.63	5.73	0.02	68.2	1.14
“ 24 hrs.	1,251	24.0	44.9	0.86	183	1.27	6.90	0.05	75.8	1.14
“ 5 days.....	1,423	69.4	51.1	2.49	225	8.21	8.48	0.31	78.2	2.40
“ 10 “	1,490	90.9	53.4	3.26	255	17.55	9.62	0.66	75.8	3.03
“ 88 “ “ complete hydrolysis.....	1,646	190.5	59.1	6.84	440	72.5	16.6	2.73	71.1	5.06
							72.4	10.5		
B. Gliadin. Total N of digest 3.816, of substrate 3.68 gm.										
Original solution...					61.7	16.2	1.67	0.44		
After 1 hr.	380	28.4	9.95	0.74	61.9	16.4	1.68	0.45	68.1	1.14
“ 24 hrs.	848	59.5	22.2	1.56	72.7	17.1	1.97	0.46	75.8	1.14
“ 5 days.....	1,105	155	28.9	4.06	96.7	19.6	2.62	0.53	78.2	2.40
“ complete hydrolysis.....							57.3	24.4		
C. Witte peptone. Total N of digest 3.551, of substrate 3.415 gm.										
Original solution...					419	12.6	12.3	0.37		
After 1 hr.	760	20.0	21.4	0.56	442	13.0	12.9	0.38	68.2	1.14
“ 24 hrs.	1,420	24.6	39.9	0.69	452	13.2	13.2	0.39	75.8	1.14
“ 5 days.....	1,710	40.1	48.1	1.13	450	15.8	13.2	0.46	78.2	2.40
“ 10 “ “ 68 “ “ complete hydrolysis.....	1,816	53.2	51.1	1.50	463	16.7	13.5	0.49	75.8	3.03
	1,970	121.5	55.4	3.42	470	38.5	13.8	1.13	72.1	4.42
							75.9	8.27		

tion, calculated upon the assumption that Witte peptone has the same nitrogen content as fibrin (16.91 per cent), was in this experiment 8.0 per cent.

RESULTS.

The immediate results of the three main experiments with their controls are recorded in Table I. This table includes also, in the "Protein control" columns, a statement of the proportions of ammonia and amino nitrogen yielded by each of the proteins

TABLE II.
Net Results of Tryptic Digestion.

Time of digestion.	Substrate nitrogen liberated.				Per cent of total hydrolysis.	
	Amount of N as		Per cent of total N as			
	NH ₂ ⁻	NH ₃	NH ₂ ⁻	NH ₃	Peptide groups.	Amide groups.
A. Casein.						
After 1 hr.....	791	7.1	29.9	0.27	44.8	2.6
" 24 hrs.....	992	21.6	37.4	0.81	56.0	7.7
" 5 days.....	1,120	58.8	42.2	2.22	63.2	21.2
" 10 ".....	1,159	70.3	43.7*	2.65	65.4	25.2
" 88 ".....	1,135	112.9	42.8	4.26	64.2	40.5
" complete hydrolysis..			(66.7)	(10.5)		
B. Gliadin.						
After 1 hr.....	250	10.9	6.79	0.30	12.2	1.23
" 24 hrs.....	700	41.3	19.0	1.12	34.1	4.6
" 5 days.....	930	133	25.3	3.61	45.5	14.8
" complete hydrolysis..			(55.6)	(24.4)		
C. Witte peptone.						
After 1 hr.....	250	5.9	7.3	0.17	11.5	2.15
" 24 hrs.....	892	10.3	26.1	0.30	41.0	3.8
" 5 days.....	1,182	21.9	34.6	0.64	54.4	8.1
" 10 ".....	1,277	33.5	37.4	0.98	58.7	12.4
" 68 ".....	1,428	78.6	41.8	2.30	65.7	29.1
" complete hydrolysis..			(63.6)	(7.9)		

upon total hydrolysis. These figures are obtained, in the cases of casein and peptone, from analyses carried out by ourselves upon representative samples boiled for 24 hours with 20 per cent hydrochloric acid; in the case of gliadin, where material for a hydrolysis was lacking, they are taken from a series of analyses by Van Slyke (15).

In order to learn, from the gross results of Table I, the net effect of the enzyme, it is necessary to deduct from the nitrogen figures of the digest the corresponding figures of both controls. When this is done we get the quantities of ammonia and free

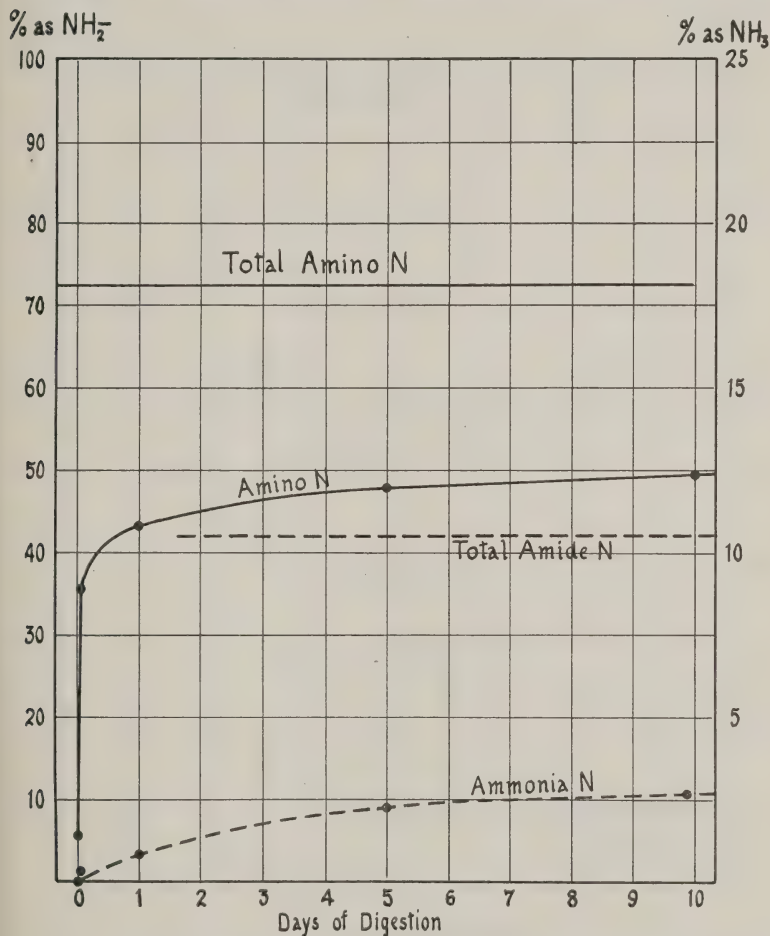


FIG. 1. Percentage of total nitrogen liberated as amino groups or ammonia during tryptic digestion of casein.

amino nitrogen which have arisen solely through the action of trypsin upon the respective substrates. These are shown in the second and third columns of Table II. In the columns

immediately adjacent the same quantities are expressed as percentages of the total substrate nitrogen, and a statement is inserted of the values which these percentages would reach in the event of total hydrolysis. These last values are calcu-

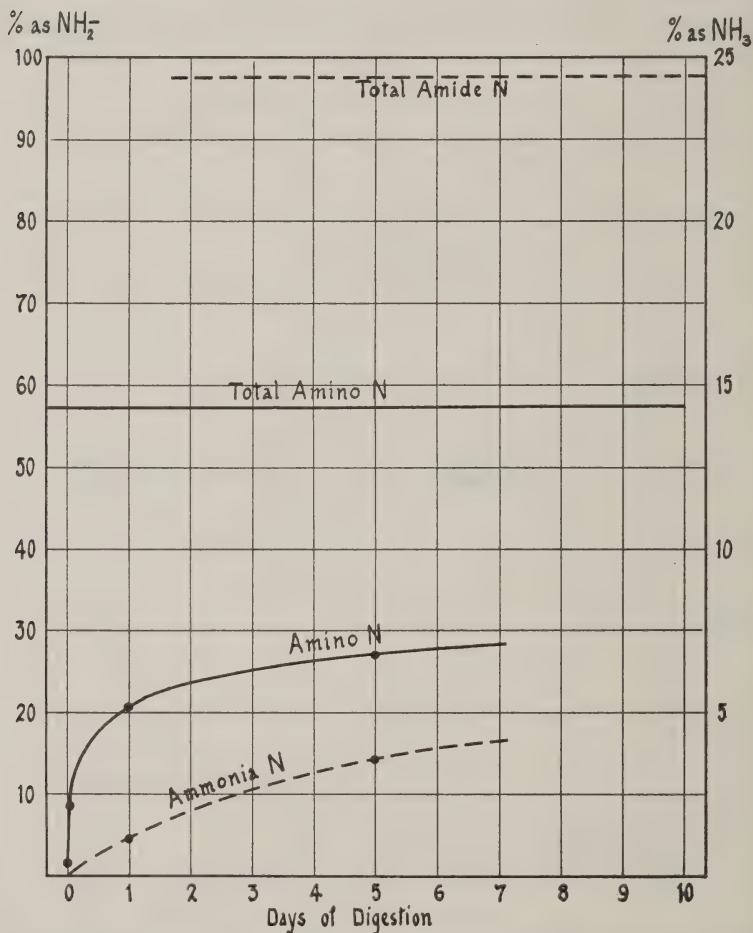


FIG. 2. Percentage of total nitrogen liberated as amino groups or ammonia during tryptic digestion of gliadin.

lated, of course, from Table I by deducting from the percentages of ammonia and free amino nitrogen in the completely hydrolyzed protein those of the corresponding fractions in the original sub-

strate. (An exception is made in the case of the ammonia from gliadin; here the value for total amide nitrogen which we took from Van Slyke is transferred from Table I to Table II without

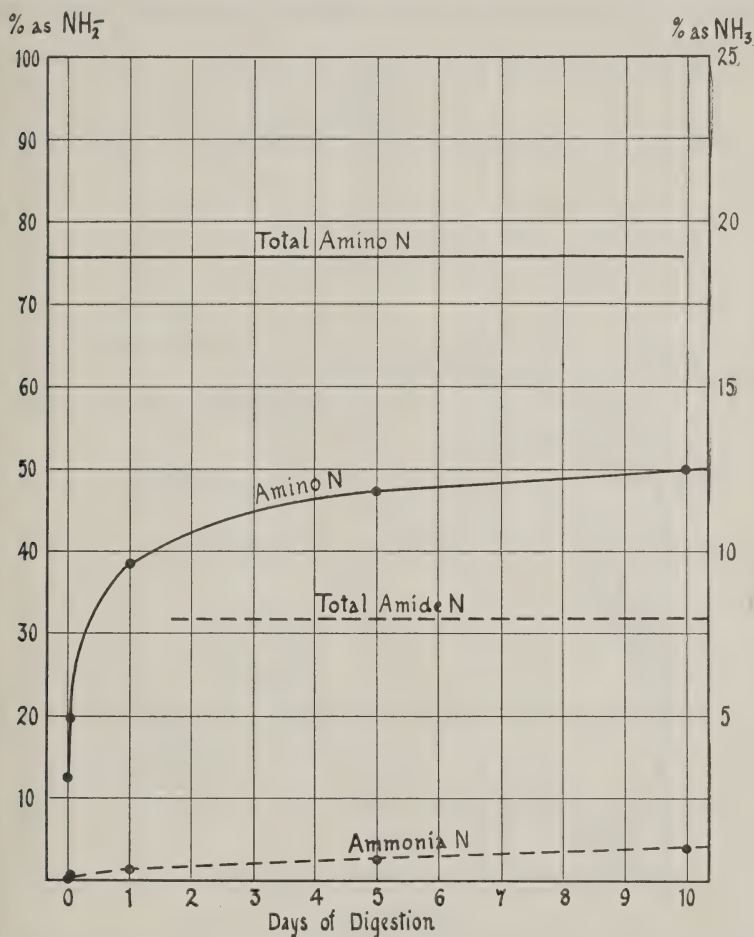


FIG. 3. Percentage of total nitrogen liberated as amino groups or ammonia during tryptic digestion of Witte peptone.

deduction, for it would be obviously improper to correct it by a determination of preexistent ammonia made by ourselves upon a different preparation.)

The data of Table II upon the percentage of substrate nitrogen

liberated in one form or the other within the first 10 days of digestion are displayed graphically in Figs. 1 to 3. In transferring them to these figures one modification has been introduced; to the percentage of nitrogen in amino form has been

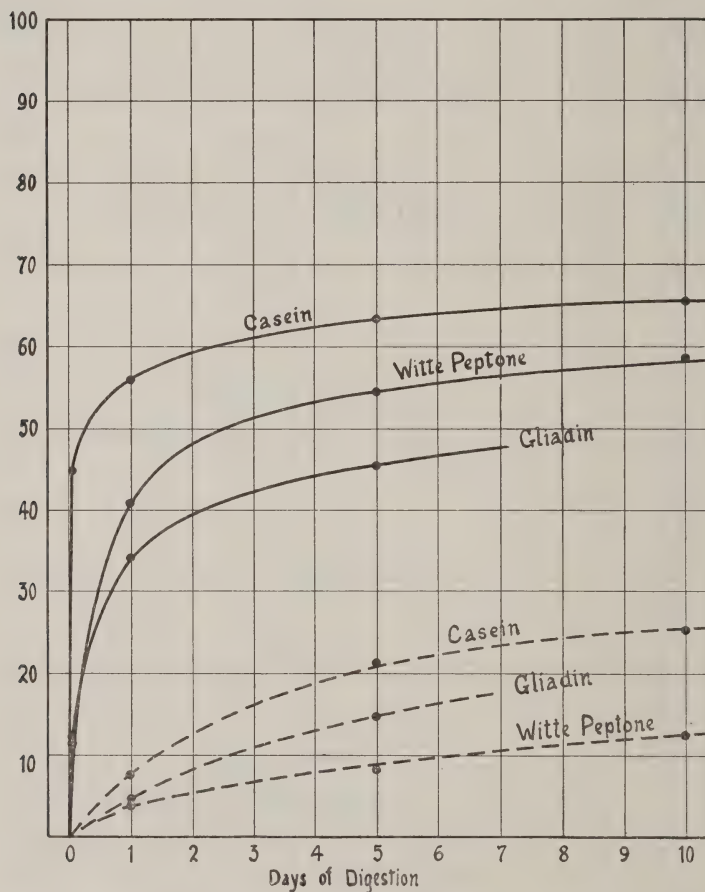


FIG. 4. Percentage of total hydrolysis in tryptic digestion. Continuous lines represent hydrolysis of peptide groups; broken lines, hydrolysis of amide groups.

added in each case the free amino nitrogen of the original protein. The amino curves represent, therefore, at each stage the total amino nitrogen of substrate origin in the digest, whether pre-

existent or liberated by trypsin, and exclusive only of that produced by the mere effect of an alkaline reaction. They start accordingly not from zero, but from 5.7 in the case of casein, 1.7 in that of gliadin, and 12.3 in that of the peptone. The scale

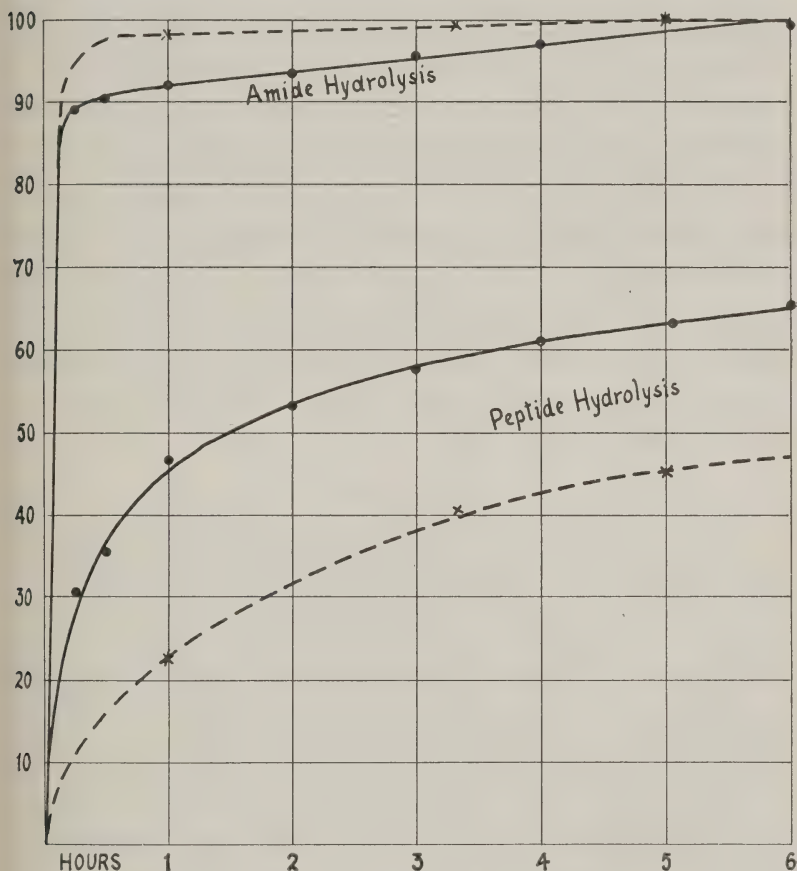


FIG. 5. Percentage of total hydrolysis of casein (continuous lines) heated with concentrated hydrochloric acid (Pittom) and of gliadin (broken lines) heated with 2.0 N hydrochloric acid (Vickery).

of reference for these curves is on the left-hand side of the charts. The ammonia results plotted upon the same scale would give curves exceedingly flat. They are, therefore, for the sake of a better display, plotted upon a scale four times as large, which

is laid out upon the right of each chart. Horizontal lines referable to their respective scales indicate the free amino and ammonia nitrogen found in the totally hydrolyzed proteins.

A statement of the quantity or proportion of "substrate nitrogen liberated" may afford a satisfactory enough basis for the comparison of different stages in the digestion of a single protein; but when, as in the present instance, we are dealing with several proteins of widely differing structure, the effect of trypsin will for many purposes be more appropriately measured by the proportion of originally existing peptide and amide linkages, that has at any given time been hydrolyzed by the enzyme. This proportion is obtained by recalculating each figure of the fourth and fifth columns (Table II) as a percentage of the corresponding figure for the state of complete hydrolysis. The results of this calculation are presented under the heading "Per cent of total hydrolysis" in the final columns of Table II. Except as they relate to days of digestion later than the 10th they are also exhibited graphically in Fig. 4.

DISCUSSION.

It is not possible in the present state of our knowledge to assign a definite limit to the proportion of peptide bonds in any one protein that may be broken through the unaided action of trypsin. The literature contains, however, a few experiments which enable us to estimate the approximate value of that limit for casein. The data of Frankel (16), for example, recalculated upon the basis used in this paper, indicate 59.7 per cent of total hydrolysis as the final effect, reached in 5 days, of a single dose of trypsin; while Dunn and Lewis (17) observed a maximal effect, after 3 days, of 52.7 per cent. In our own casein experiment hydrolysis, as judged by the amino nitrogen, was even more rapid and extensive than in either of these examples. Our ferment therefore possessed a highly satisfactory degree of activity, and was capable in all probability of carrying the peptide hydrolysis of casein nearly, if not quite, to the attainable limit of tryptic action.

The point is emphasized because it shows that the ammonia results, upon which our attention was particularly directed,

must be regarded as those associated with full tryptic activity. Now it requires but a glance at the ammonia data of Table II or at its accompanying graphs to show that amide hydrolysis falls far behind peptide hydrolysis not only in absolute velocity (Figs. 1 to 3) but (what is more to the point) in the relative speed with which the state of total hydrolysis is approached (Fig. 4). Thus, after 10 days' digestion, at a time when, as appears from Table II, 65 per cent of its bound amino nitrogen has already been liberated, casein has yielded but a quarter of its total potential ammonia. Even more striking is the circumstance that the degree of peptide hydrolysis attained by this protein within the very 1st hour (44.8 per cent) is not quite equalled by the ammonia production of 88 days. Gliadin, on account doubtless of the mere mass action of its more numerous amide groups, gives larger absolute amounts of ammonia than casein, but it falls still further short of total amide hydrolysis. Witte peptone yields in a given time less ammonia, relatively as well as absolutely, than either of the other substrates; from which incidentally it may be inferred that a previous peptic digestion does not render the amide linkages more susceptible to the action of trypsin. All in all, the amidolytic, as distinguished from the peptolytic, activity of the enzyme is shown to be exceedingly feeble.

One might be tempted to explain the contrast between the amino and the ammonia curves by supposing that only a relatively small fraction of the total amide nitrogen is at all susceptible to the action of trypsin; that both sets of curves are simply approaching their respective maxima at proportionate rates; and that they will finally reach these in identical times. This, it can easily be shown, is not the case. The free amino nitrogen of the casein digest increases somewhat between the 10th and the 88th day (Table I); but the net results of Table II show that this increase is fully accounted for by spontaneous hydrolysis of the substrate, and is in no degree due to the specific activity of the enzyme. By the 10th day therefore, if not earlier, trypsin has definitely ceased to effect any further liberation of amino groups. On the other hand, the enzymatic production of ammonia continues at such a rate that the 88th day marks a gain of 61 per cent over the 10th. With the peptone experiment the case, though not identical, is analogous. Here there is

a real net gain in amino nitrogen after the 10th day; but it amounts, in 58 days, to less than 12 per cent of the quantity already liberated, and probably denotes a maximum actually attained much earlier. During the same 58 day period the ammonia is considerably more than doubled. In the case of gliadin we made no observations later than the 5th day, but if we had we should doubtless have found similar relations. It would seem therefore that at the time, reached comparatively soon, when the characteristic trypsin effect—peptide hydrolysis—is practically exhausted, the production of ammonia is still in active progress. How long this progress may continue, and what proportion of the total amide nitrogen may ultimately be liberated, our experiments do not reveal; but, if the graph of Fig. 4 be expanded so as to include the final observations upon casein and peptone, neither curve will be found even then to indicate the approaching cessation of amide hydrolysis. According to Luck (11) the final state, at least for casein, would be one in which about 66 per cent of the amide groups had been split off.

The absence of relation, in our experiments, between peptolysis and amidolysis is so conspicuous that these processes would really seem to have been catalyzed by two separate enzymes. We venture accordingly to suggest, as a working hypothesis, that the liberation of the amide nitrogen of proteins is not, strictly understood, a function of trypsin at all, but is to be attributed to the action of a specific enzyme, possibly of tissue origin, by which trypsin as usually prepared is liable to be accompanied.

A certain amount of indirect support for this view is to be found in the difference between our results and some of Henriques and Gjaldbæk's (6). These workers, to be sure, used a very weak preparation of trypsin, determined ammonia and amino nitrogen by formol titration, and carried out no controls. Their experimental conditions, therefore, were very different from ours, and their data may be compared at best only with those to be found under "Protein digest" in Table I. The comparison is none the less instructive. In an experiment in which casein was digested for 21 days with trypsin alone Henriques and Gjaldbæk found ammonia to represent 5.0 per cent of the total nitrogen, while the formol-titratable nitrogen was 40.0

and the amino nitrogen accordingly 35.0 per cent; but at a slightly later stage of peptide hydrolysis (reached, according to Table I, in a single hour) our casein digest showed an ammonia nitrogen of only 0.32 per cent. In the case of gliadin (19 days) Henriques and Gjaldbæk found with 22.8 per cent of amino as much as 9.1 per cent of ammonia nitrogen, and in that of Witte peptone (21 days) with 38.7 per cent of amino 3.3 per cent of ammonia nitrogen; at approximately the same stages of digestion (after 24 hours) we found only 1.56 per cent of ammonia nitrogen for gliadin and 0.69 per cent for peptone. These differences may be partly, but cannot be altogether, due to differences of technique. It would seem, therefore, that, while the trypsin used by Henriques and Gjaldbæk was much less active than ours in peptide hydrolysis, it was relatively (and even absolutely) a good deal more potent in the liberation of amide groups. There are but two ways of accounting for such a difference. Either, as already suggested, we are dealing here with two separate enzymes, which may occur in pancreatic extracts in varying proportions; or else trypsin is an enzyme of variable properties, the diverse activities of which stand in no fixed ratio to one another. Of these alternatives the former is the one which for the present we prefer to accept. A final choice between them, or an escape from the apparent dilemma, might be sought by studying the ammonia-producing power of pure pancreatic juice or of "trypsins" that have been subjected to different methods of purification.

Whether the results obtained in our experiments are due to the action of one enzyme or of more, the order of events observed offers a great contrast to that presented during the hydrolysis of proteins by acids. This is made evident by a comparison of Fig. 4 with Fig. 5. In the latter we have utilized certain data of Pittom (1) and Vickery (2) to show the relative rate of hydrolysis of amide and peptide groups when casein or gliadin is heated with hydrochloric acid. It will be seen that, whereas in enzyme hydrolysis the peptide linkages are split at a very much greater rate than the amide linkages, under the action of hydrogen chloride the outcome is reversed; for in the acid hydrolysis the ammonia has been all but completely liberated at a time when only 25 to 50 per cent of the bound amino nitrogen has become free.

This discussion would be incomplete without a further reference to the recent work of Luck (11) which, although dealing solely with casein, has an important bearing upon the general topic of this paper. Luck noticed that even prolonged hydrolysis of casein with a pancreatic extract failed to liberate the entire quota of amide nitrogen. Following up this observation he found that the production of ammonia in tryptic digestion ceased altogether when, after an interval of 12 to 56 days, about two-thirds of the theoretical total had appeared. This implied the existence in the casein molecule of amide combinations not merely resistant but completely stable to trypsin. From the final product of digestion Luck actually isolated, in a state of relative purity, a trypsin-stable complex still retaining about one-third of the original amide nitrogen. As far as the relative resistance of the amide groupings to tryptic hydrolysis is concerned, Luck's results are in general confirmatory of our own, while his demonstration of the final cessation of ammonia production is in no way irreconcilable with the tentative conclusion we have drawn from our experiments.

The results obtained with "trypsin" raise naturally the question whether the amide groups of proteins are attacked by other proteolytic enzymes. We have already attempted a few experiments with erepsin which indicate, as far as they go, that that enzyme is no more efficacious than trypsin in the production of ammonia from peptone. Unfortunately the erepsin preparations were not very active. We propose, therefore, before forming a definite conclusion, to repeat these experiments under more satisfactory conditions.

SUMMARY.

We have compared the rate of liberation of ammonia with that of amino nitrogen in casein, gliadin, and Witte peptone subjected to the action of commercial trypsin. In all three cases amide hydrolysis was found to take place very much less rapidly than peptide hydrolysis. The differences were so great as to suggest that the two processes are really catalyzed by different enzymes, and that pure trypsin might produce from proteins no ammonia at all.

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ON THE RELATIVE PROPORTIONS OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN BLOOD PLASMA IN RENAL DISEASE.*

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Since the introduction of microchemical methods for the estimation of sodium, potassium, calcium, and magnesium in blood (1915), a fair volume of literature has accumulated with particular reference to the part that alterations in the concentration of these elements may play in disease. Practically no consideration has, however, been given to the possible influence of another form of disturbance; namely, the alteration, not only in concentration, but in the relative proportions of the individual constituents.

Before the extensive studies of Macallum (1903) very few analyses of the inorganic composition of body fluids were made and the biological importance of such a study was not fully appreciated. In the invertebrate kingdom, only the Crustacea, Mollusca (Griffiths, 1892), and *Limulus polyphemus* (Genth, 1852; Gotch and Laws, 1885) were studied. Mammalian plasma, including that of the ox, pig, horse, and dog, was analyzed by Bunge (1876) and that of the sheep, goat, rabbit, cat, horse, ox, pig, and dog by Abderhalden (1897). Schmidt (1850) first recorded the analyses of human serum.

From the result of a study of the inorganic composition of the Medusæ, *Aurelia* and *Cyanea*, Macallum (1903) concluded that in many invertebrates with a vascular system still freely communicating with the exterior, the circulating fluid is sea water. The same author then advanced the view that the blood plasma

* This work was done with the aid of a grant from the Cooper Memorial Fund.

of vertebrates and invertebrates with a closed circulatory system is, in its inorganic salts, but a reproduction of the sea water of the remote geological period, in which the prototypic representatives of such animal forms first made their appearance. Thus in comparing the values he found for blood serum with those of ocean water previously determined (Dittmar, 1873-76) the following results were obtained:

	Na	K	Ca	Mg
Blood serum.....	100	6.69	2.58	0.8
Ocean water.....	100	3.66	3.84	11.99

In a subsequent communication Macallum (1904) amplified these observations and explained the discrepancy in the magnesium values. In 1910 the same author made a series of analyses on vertebrates and invertebrates with a closed system, including the *Gadus callarias* (cod), *Pollachius virens* (pollock), *Limulus polyphemus* (horseshoe crab), *Homarus americanus* (lobster), *Acanthias vulgaris* (dogfish), and the whale. In this investigation he found a uniformity of the composition of the internal medium and concluded that this uniformity was a powerful factor influencing the course of evolution. "The capacity of the organism to make and keep its own internal medium uniform gives an enormous advantage to it, for it can change its habitat and adapt itself to a new environment without affecting the stable conditions under which its own tissues and organs do their best work. The organ which enables the organism to maintain these paleo-oceanic conditions is the kidney and this was its first function. The firmly fixed physiological habit or function must be the more ancient one, and consequently the earliest function was not the elimination of waste metabolic products, but the regulation of the inorganic composition of the blood."

The possible application of these observations to clinical medicine appears to have been overlooked, since no study of the blood plasma from this point of view, in conditions in which the function of the kidney is regarded as impaired, was made until the same author (1917) recorded his results in four cases of eclampsia. These showed that at least temporarily in eclampsia

Hospital No.	Concentration.				Ratios.				Urea N. <i>mg. per 100 cc.</i>	Creatinine. <i>mg. per 100 cc.</i>	Clinical diagnosis.
	Na	K	Ca	Mg	Na	K	Ca	Mg			
3673	320	45.18	6.912	2.048	100	14.26	2.16	0.64	250	9.09	Chronic nephritis; uremia; myocarditis.
6325	314	71.68	5.746	2.166	100	22.3	1.83	0.69	237	7.10	"
4245	318	19.58	8.268	2.575	100	6.16	2.60	0.81	211	1.20	"
927	326	19.98	6.614	2.738	100	6.13	2.03	0.84	203	7.89	"
1987	321	19.54	8.988	2.921	100	6.09	2.80	0.91	196	8.10	"
4526	332	20.51	8.930	2.988	100	6.18	2.69	0.90	168	5.70	"
3808	299	18.86	8.162	2.182	100	6.31	2.73	0.73	168	1.20	"
5644	294	18.54	8.290	2.469	100	6.24	2.82	0.84	150	7.14	"
1071	342	34.09	6.805	2.804	100	9.97	1.99	0.82	147	6.66	"
2549	304	21.70	6.110	2.492	100	7.14	2.01	0.82	138	2.34	myocarditis.
3441	298	26.93	8.016	2.354	100	9.04	2.69	0.79	138	4.70	"
90-24	334	21.04	6.553	2.404	100	6.30	1.96	0.72	116	3.23	"
1347	295	18.31	7.729	2.242	100	6.21	2.62	0.76	105	4.41	"
4288	324	19.63	8.942	2.494	100	6.06	2.76	0.77	90	2.74	myocarditis.
6445	341	23.90	11.04	2.352	100	7.01	3.24	0.69	50		"
1360-23	310	19.25	7.872	1.612	100	6.21	2.54	0.52	43	1.72	"
1746	336	20.19	8.260	3.057	100	6.01	2.46	0.91	39	1.41	"
5857	334	20.50	11.42	2.572	100	6.14	3.42	0.78	38		"
E 2	337	20.99	8.290	2.156	100	6.23	2.46	0.64	36	1.84	Eclampsia.
E 1	318	58.00	8.331	2.575	100	18.24	2.62	0.81	32	1.76	"
2618	318	19.01	7.094	2.194	100	5.98	2.86	0.69	29	1.42	Chronic nephritis; myocarditis.
6273	321	20.15	12.87	2.889	100	6.28	4.01	0.90	24		"
K p.v.*	319	20.03	7.943	2.169	100	6.28	2.49	0.68	21	1.12	Polycythemia vera.
P p.v.*	327	19.97	8.240	2.746	100	6.11	2.52	0.84	20	1.03	"
D. Ac.†	184	11.33	5.538	0.772	100	6.16	3.01	0.42			Diabetic coma. Chronic nephritis.

* Polycythemia vera.

† Acidosis.

the kidneys concerned in maintaining the normal ratio of potassium in the blood plasma suffer a partial or total suppression of function. In the course of a study of renal function observations were made by the writer from the above point of view. The results are of sufficient interest to justify their publication.

Material.

The data were obtained from the analyses of twenty-three patients suffering from advanced renal or cardiac disease. An opportunity was afforded to make observations on two cases of polycythemia vera and these are also tabulated. The combined data are recorded in Table I. Under the heading of clinical diagnosis only the predominant clinical features are recorded. The data are recorded, not in order of the time of admission of the patients to the hospital, but in order of the degree of impairment of renal efficiency, as indicated by the concentration of non-protein nitrogenous elements (urea and creatinine) in the blood.

Methods.

In the experience of the writer, the microchemical methods for the estimation of Na, K, Ca, and Mg were found unsatisfactory. That he is not alone in this experience is evident from the literature. Since Hamburger (1915) first attempted to employ small quantities of fluid for such determinations, no less than twenty-one different modifications are recorded for the various elements. The methods followed in this investigation were those employed by Macallum in his original communications with slight modification, and they are as follows:

Estimation of Sodium and Potassium.

The fluid (30 to 40 gm.) is weighed in a platinum crucible or capsule, evaporated on a water bath to dryness, and the evaporation completed in an oven at a temperature of about 110°C. for 7 to 8 hours. After cooling in a desiccator, the preparation is weighed and the percentage of solids in the fluid determined.

The crucible or capsule is now heated over a Bunsen flame to a temperature that causes the fumes to arise from the residue. These latter should catch fire and burn above the residue. The heat is continued at this low temperature until all the volatile matter is gone. Then the residue is

kept at a *dull red heat* for varying periods according to the mass of the residue in order to carbonize it completely.

The carbonized residue is now extracted repeatedly with hot water and the extracted fluids are all combined. The residue is now dried and made to undergo complete combustion, and the ash set free is treated with dilute hydrochloric acid, the resulting fluid filtered, and the filtrate added to the combined extraction fluids.

The latter should be evaporated, on a water bath, to about 50 cc. To it is then added a saturated solution of baryta which precipitates all the phosphoric and sulfuric acids as phosphate and sulfate of barium. These are filtered off and the filtrate, received into the platinum capsule, is evaporated down to dryness and the residue fused completely with water-free oxalic acid.

The fused residue is now dissolved in the smallest possible quantity of water (2 to 3 cc.), filtered through the smallest possible filter into a platinum capsule, and the filtrate evaporated down to dryness, carefully heated over a Bunsen flame, and dissolved once more in 2 to 3 cc. of water. If the resulting solution is not clear, it must be filtered again. When clear, a small quantity of hydrochloric acid is added to it, and it is evaporated to dryness. The residue consists now wholly of the chlorides of sodium and potassium, which after being heated in the capsule to dull redness and then cooled may now be weighed.

To determine how much potassium and sodium there is in the mixture one adds an approximately calculated quantity of 10 per cent platinum chloride solution to the solution of the two salts, and the mixture is evaporated down, with frequent stirring, to dryness on a water bath. If sufficient platinum chloride is added then all the potassium chloride is in combination with it. The remainder of the platinum chloride enters into combination with the sodium chloride.

On the dried double salts one places 20 to 30 cc. of absolute alcohol and allows the preparation to stand under cover for an hour. Then to this is added 10 to 15 cc. of ether, and the mixture is then allowed to stand for a further hour.

The mixture of ether and alcohol is then carefully decanted from the residue and a fresh quantity of alcohol and ether (20 to 30 cc. of alcohol and 10 to 15 cc. of ether) is added and allowed to stand for another hour, then decanted, and a fresh quantity is poured on for another hour. The residue is then thoroughly dried at room temperature and the platinum in it reduced to the metallic condition by heating in hydrogen to 250°C.

The residue is then treated with water, the platinum filtered off, the filter containing it burned, and the metallic platinum weighed. From this weight can be calculated the quantity of potassium present, also the quantity of potassium chloride, and by subtracting the thus calculated amount of the latter from the combined weights of the two chlorides, the quantity of the sodium chloride may be ascertained.

Estimation of Calcium and Magnesium.

To the weighed quantity of plasma (25 to 30 gm.), placed in a platinum dish, about 2 gm. of pure sodium carbonate are added; the moisture, after being carefully stirred, is evaporated to dryness, the residue carbonized, then extracted several times with hot water, acidulated with hydrochloric acid, the remainder of the residue completely incinerated, the ash extracted with hot dilute hydrochloric acid, the fluid filtered, and the filtrate added to the volume of the united filtrates previously obtained.

The united filtrates are then treated with crystals of ammonium oxalate and ammonia, and after standing 24 hours the calcium oxalate precipitate is removed, incinerated to a constant weight, and weighed as CaO. From the filtrate the magnesium is precipitated as magnesium phosphate by the addition of ammonium phosphate and ammonia.

DISCUSSION.

The average normal ratios as recorded by Macallum are as follows: Na, 100; K, 6.11; Ca, 2.71; and Mg, 0.85. It will be noted that in only five of the twenty-three cases of cardiorenal disease could it be definitely stated that there was a disturbance in ratios of any of the elements. Both from the clinical diagnosis and blood urea nitrogen data it is obvious that in more than half of these cases (fourteen) there was a marked impairment of renal efficiency. All these patients died. With the exception of the one case of eclampsia in which a disturbed ratio was found, the remainder of disturbed ratios are all found in the advanced (uremic) cases. That there were nine other such cases which showed normal ratios in spite of the advanced renal lesions appears to emphasize further the fundamental nature and primary importance of this function of the kidney. An observation which may by further studies be found significant is that in each of the five cases with the high potassium ratios, the heart at autopsy (Dr. L. J. Rhea) was found markedly dilated. The electrocardiographic tracings (Dr. C. C. Birchard) in these cases were normal, in as far as the spread of excitation wave over the ventricles, duration of refractory period, and conduction interval of the bundle of His were concerned.

I wish to express my sincere thanks to Prof. A. B. Macallum for his kind interest, and also to Miss Althea Frith for assistance in this work.

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THE ENZYMATIC SYNTHESIS OF PROTEIN. III.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION ON PEPTIC SYNTHESIS.

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In two previous communications the authors have described a synthesis of protein by pepsin in a concentrated peptic hydrolysate of albumin (1); and the effect of temperature on this synthesis (2). The justification for describing the synthetic product as protein is discussed in our previous paper (2). In these communications the optimum hydrogen ion concentration was stated to be pH 4.0; but the influence of the hydrogen ion concentration was otherwise not discussed. The importance of the degree of acidity was realized early by Sawjalow (3), who did not, however, define it precisely, and by Henriques and Gjaldbæk (4), who gave the optimum pH as 1.5. This hydrogen ion concentration in our experience, despite the existence of all other optimum conditions, allows only very small amounts of synthesis. Either Henriques and Gjaldbæk were in error, or the occurrence of the optimum pH at 1.5 was due to some as yet unrecognized factor.

In order to investigate the influence of hydrogen ion concentration on protein synthesis, the pH was altered while other conditions were kept constant, and the resulting changes in the amount of protein synthesized were then observed.

Various amounts of acid and alkali were added and the resulting pH in each case ascertained electrometrically. The mixtures were then adjusted in such a manner that the dilutions were in each case the same, and the pH was again checked. The pH given in Table I is this final value. In these determinations of the hydrogen ion concentration, a Moloney electrode was employed (5).

From each mixture two 20 cc. portions were then pipetted into 50 cc. Erlenmeyer flasks. To each was added 1.0 gm. of pepsin (Eimer and Amend), which was dissolved in the digest by rubbing with a stirring rod. After adding 0.5 cc. of chloroform to each, the flasks were tightly stoppered and set away at 37.5°C. for 2½ days. They were then removed, neutralized with 50 per cent NaOH to stop further peptic action, diluted to 100 cc., and set away in an ice chest at 13°C. It was not possible to analyze any of the samples immediately, nor all of them simultaneously, so that the length of stay in the ice chest was not the same for all samples. The number of days intervening between neutralization and analysis is noted in the column headed "Time of standing" (Table II).

TABLE I.

Digest.	5 N HCl.	50 per cent NaOH.	H ₂ O	pH
cc.	cc.	cc.	cc.	
45	2.94	0.00	1.8	0.9
45	1.35	0.00	3.6	1.8
45	0.00	0.00	4.9	2.2
45	0.00	1.90	2.84	3.2
45	0.00	3.81	0.93	4.0
45	0.00	4.74	0.00	4.7

TABLE II.

pH	Series 1.		Series 2.	
	Time of standing.	Protein N in per cent of total N.	Time of standing.	Protein N in per cent of total N.
	<i>days</i>		<i>days</i>	
0.9	24	1.3	32	1.0
1.8	23	3.2	32	1.4
2.2	16	7.1	32	6.9
3.2	16	17.2		
4.0	11	27.3	28	23.3
4.7	11	18.7	28	16.0

The amount of protein synthesized was estimated by precipitation from the neutral diluted solution with trichloroacetic acid and is the difference between the total nitrogen contained in the solution before addition of the trichloroacetic acid and after filtering off the precipitate.

Very soon after dissolving the pepsin an increase in the opacity and viscosity was observed in the solutions. In the two most acid solutions (pH 0.9 and 1.8), the increase in turbidity was

only faint and the viscosity was not noticeably greater than in the control containing no enzyme (pH 4.0). The remaining solutions exhibited gradually increasing opacity and viscosity, progressively greater as the acidity of the solutions diminished. That at pH 2.2 was intermediate in opacity, 3.2 was densely opaque but still somewhat fluid, 4.0 and 4.7 were densely opaque and stiff immovable jellies. 2 hours later the same relationships were maintained, except that a slight precipitate had appeared in pH 1.8.

In Table II, Series 1 is the series first analyzed, *i.e.* which remained in the ice chamber the shorter period; Series 2 is the duplicate of Series 1, except that the samples were analyzed later.

Neither metaprotein nor trichloroacetic acid precipitable material other than insoluble protein could be detected in any of the experiments represented in Table II.

The optimum pH for protein synthesis in a peptic digest of egg albumin is at pH 4.0 (Tables II and IV). On standing at 13°C. for long periods some hydrolysis of the protein formed occurs. This is due not impossibly to a tissue enzyme which may be contained in the pepsin preparation and which is, as shown by Bradley (6), not killed at pH 2.0.

The figures obtained almost certainly represent equilibrium amounts and not velocities. This was clearly indicated in our earlier work, before the optimum hydrogen ion concentration had been discovered. Samples were set away at 38°C. at pH 1.7 for a number of days, and the synthesis followed from day to day (Table III). The concentrations of material were approximately the same, and the results are comparable with the results given above. It is clear that equilibrium is attained in approximately 2 days, even under suboptimal conditions.

The cause of the subsequent decrease shown in Table III is obscure.

A series was now carried out with most of the points on the alkaline side of pH 4.0. A concentrated, new peptic digest and Merck's pepsin, which gram for gram was found to be more potent than the pepsin previously used, were employed. The series was incubated for the usual period of 3 days at 38°C., after which the solutions were neutralized, diluted, and analyzed immediately for protein.

The optimum hydrogen ion concentration was again found to

be at pH 4.0; but the maximum amount of protein synthesized was higher than in the previous experiment. The results over the whole range are given in Table IV. They are the averages of duplicates whose maximum variation was of the order of magnitude of ± 1 per cent of total nitrogen.

These figures, like those in Table II, represent equilibrium amounts. Mixtures incubated for 6 days showed no significant differences over those incubated for only 3 days; *e.g.*, one sample,

TABLE III.

Days.	Protein N in per cent of total N.
$\frac{3}{4}$	3.3
$1\frac{5}{8}$	5.1
$6\frac{5}{8}$	4.4
$10\frac{1}{2}$	3.2
$13\frac{1}{2}$	2.4

TABLE IV.

pH	Protein N in per cent of total N.
2.8	20.2
3.4	31.0
4.0	33.7
4.3	31.9
4.45	32.1
4.9	30.7
5.45	13.6
5.6	13.9
6.0	5.9
6.6	3.3

similar to the mixture at pH 6.6 (Table IV), incubated for 9 days, gave 3.4 per cent of protein.

The percentages of the total nitrogen combined in the protein were calculated on the basis of the nitrogen content of the digest alone; *i.e.*, the added pepsin nitrogen was deducted from the total nitrogen of the mixture. The justification for this seemingly arbitrary procedure was found in the fact that a 24 per cent solution of pepsin at pH 4.0, maintained for weeks at 38°C., showed no signs of any formation of protein, notwithstanding that at the end of this period, both the synthesizing and hydrolyzing powers of the solution were found to have remained unimpaired.

In order to observe more easily the effect of acidity over the whole range in which protein synthesis occurs, the results in Tables II and IV were combined. These results, having been obtained on different digests and with different enzyme preparations, show considerable variation. They were therefore recalculated so that the maximum amount of protein synthesized in each case at pH 4.0 was taken as 100 per cent, and the lower amounts in each experiment as the corresponding fractions of this value (Table V). The resulting figures were plotted as ordinates, against the pH values as abscissæ.

TABLE V.

Protein Synthesized at Various Hydrogen Ion Concentrations.

pH	Actual value per cent of total N.	Per cent of maximum.
0.9	1.3	4.7*
1.8	3.2	11.7*
2.2	7.1	26.0*
2.8	20.2	60.0†
3.2	17.2	63.0*
3.4	31.0	91.9†
4.0	27.3	100.0*
4.0	33.7	100.0†
4.3	31.9	94.6†
4.45	32.1	95.3†
4.7	18.7	68.5*
4.9	30.7	90.1†
5.45	13.6	40.4†
5.6	13.9	41.3†
6.0	5.9	17.5†
6.6	3.3	9.8†

* Calculated on the basis of 27.3 per cent as maximum.

† Calculated on the basis of 33.7 per cent as maximum.

The curve obtained resembled in form and slope either the primary dissociation curve of a dibasic acid or the curve representing the undissociated residue of an amphoteric electrolyte.

To ascertain the degree of correspondence the curve was considered as such and the values for the primary and secondary dissociation constants, pK_1 and pK_2 , or, in the alternative, the acid and basic dissociation constants of an ampholyte (pK_a and pK_b) were obtained by the tangent method of Michaelis.¹ These

¹ Michaelis (7), p. 64.

were found to be 2.67 and 5.33 for pK_1 and pK_2 , and 5.33 and 11.33 for pK_a and pK_b .

The constants found were inserted into the formula given by Michaelis² and the degree of primary dissociation α and the magnitude of the undissociated residue ρ were calculated for pH intervals of 0.5. These calculated values, identical, of course, for α and ρ , are recorded in the first column of Table VI. In the second column of the same table the maximum value of α and ρ (91.4 per cent) is set as 100 and the other values readjusted to that scale and recorded as α_1 or ρ_1 . This is done in order to facilitate a comparison with the observed amounts of synthesis obtained at

TABLE VI.
Values of α and ρ .

pH	Calculated α (ρ).	Modified α_1 (ρ_1).
1.0	2.1	2.3
1.5	6.6	7.2
2.0	17.6	19.3
2.5	40.3	44.1
3.0	68.0	74.4
3.5	86.0	94.1
4.0	91.4	100.0
4.5	86.0	94.1
5.0	68.0	74.4
5.5	40.3	44.1
6.0	17.6	19.3
7.0	2.1	2.3

various hydrogen ion concentrations which have also been expressed (Table V) as percentages of the maximum. On Fig. 1 a graphic comparison is obtained. The surprising approximation of the theoretical dissociation to the extent of synthesis is evident.

The hydrogen ion concentration in which primary ionization is a maximum is given by the equation $[H^+] = \sqrt{K_1 K_2}$. From pK_1 and pK_2 , 2.67 and 5.33 respectively, the theoretical position of the maximum is at pH 4.0. Again, the isoelectric point of an ampholyte is given by the equation $I = \frac{\sqrt{K_a}}{K_b} K_w$ which, for $K_a = 5.33$ and $K_b = 11.33$, gives the isoelectric point at pH 4.0. This point is precisely the position of the experimental optimum.

² Michaelis (7), pp. 26 and 36.

The values for pK_1 and pK_2 do not correspond to the published dissociation constants of any of the known diamino or dicarboxylic acids, nor do the values for pK_a and pK_b correspond to the pub-

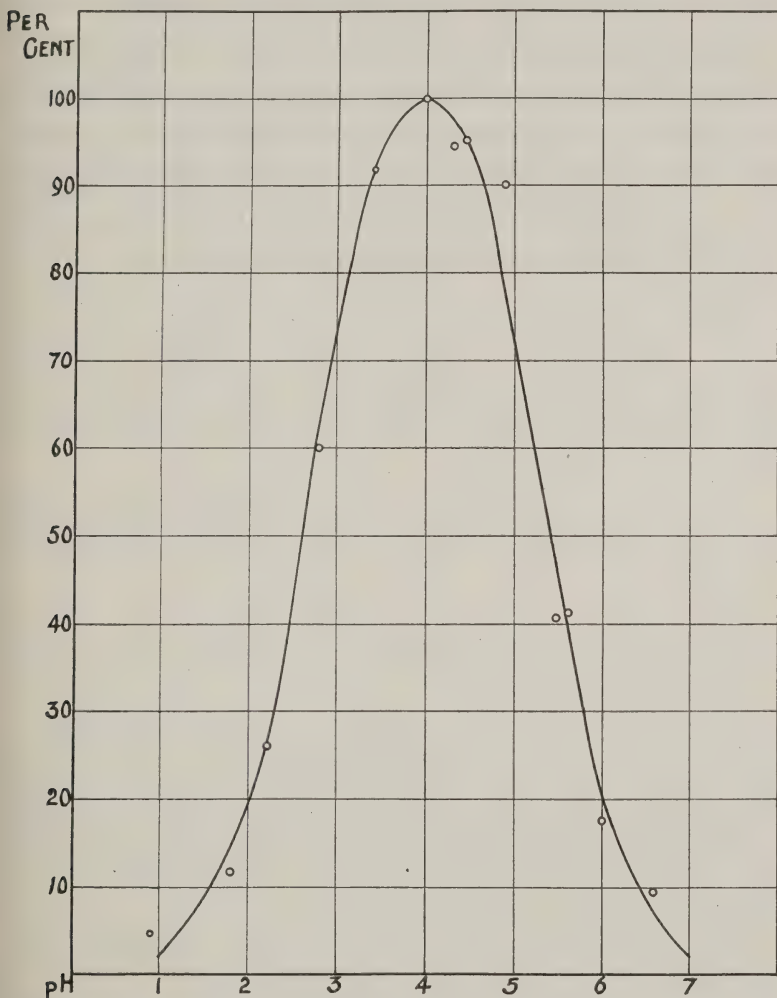


FIG. 1. Relation between pH (abscissæ) and amount of protein synthesized (ordinates). Curve represents calculated values of α_1 (or ρ_1) (Table VI). Points indicated o are the experimentally obtained protein values (Table V). Experimental points represent the average of two or more determinations.

lished dissociation constants of any amino acid. It is not impossible, however, that Fig. 1 may represent the dissociation of one of these amino acids as it exists in the concentrated digest, with the values for the constants somewhat modified by the presence or manner of its combination with other groups.

The importance of the above correspondence between the experimental values and the calculated dissociation curve rests in the conclusion drawn from it that the amount of protein synthesized at any given hydrogen ion concentration is dependent upon the degree of ionization of a component of the digest, *i.e.* the substrate, for electrometric titration curves showed quite unmistakably that the ionization of the digest, taken as an entity, was not represented by Fig. 1.

At the outset the effect of varying the hydrogen ion concentration might have been sought in variations brought about in the functional condition of the enzyme. There were two possibilities; the varying acidity affected either the rate of autodestruction or the ionization of the pepsin.

Assuming the first possibility, the optimum pH, then, is simply that degree of acidity in which synthesis proceeds to the greatest extent before it is brought to a stop by the disappearance of active enzyme.

The known facts regarding the autodestruction of pepsin do not support this hypothesis. Michaelis and Rothstein (8) and Morgenroth (9) have found that in 10 per cent NaCl, pepsin solutions at approximately pH 3.3 may stand at room temperature for a week without suffering any autodestruction, and in glycerol for more than a year. Our results are confirmatory, and it seems certain from extensive investigation in this laboratory that at acidities greater than pH 6.0 the autodestruction of pepsin cannot be a factor in determining the location of the optimum pH for peptic synthesis.

The alternative possible effect of acidity, *viz.* its influence upon the degree of ionization of the enzyme, remains.

In view of the previous result that the effect of pH on the amount of synthesis can be represented graphically by a dissociation curve, it follows, if the dissociation under consideration be the dissociation of the enzyme, that a direct linear proportionality must exist between concentration of active enzyme and extent of

synthesis. It is found, however, that the relationship between the equilibrium amounts of protein and the enzyme concentration is not linear, but exponential, of the type $Y = AX^n + B$.

We conclude that, in a broad sense, it is through the ionization of the substrate that the hydrogen ion exerts its influence on the peptic synthesis of protein. The steepness of the dissociation curve seems to preclude any possibility of accidental coincidence in the correspondence of the experimental values to the calculated curve. A similar explanation has been proposed by Northrop (10) for the location of the optimum pH of peptic hydrolysis.

That there should be any dependence of the equilibrium quantities of protein synthesized on the concentration of the pepsin was a surprising result, though indications of the same phenomenon are to be found in the experimental results of Sawjalow (3) and of Robertson (11).

TABLE VII.

Digest No.	0.1 HCl added.	Enzyme (Merck).	Concentrated digest at pH 4.0.	Total N.	N after filtration.	Protein N.	Protein N in per cent of total N.
	cc.	gm.	cc.	mg.	mg.	mg.	
1	1	0.01	10	857	744	113	13.1
2	1	0.05	10	848	681	167	19.7
3	1	0.10	10	858	661	197	23.0
4	1	0.20	10	843	615	228	27.1
5	1	0.40	10	837	584	253	30.1
6	1	0.80	10	839	560	279	33.2

In attempting to determine this effect of enzyme concentration on the extent of synthesis the procedure adopted was the same as that used in the previous experiments. Six mixtures were made with relative enzyme concentrations varying over a range from 1 to 80. The pH and the concentration of the digest were unchanged. The full details and the results are given in Table VII.

Enzyme action was allowed to continue for 36 hours at 37°C. The samples were then neutralized, diluted to 100 cc., and analyzed for protein in the usual manner. The figures for total nitrogen are the values for the digest alone, *i.e.* the nitrogen content of the added pepsin subtracted from the figure actually obtained.

The curve for protein formation plotted against enzyme concentration is of the type of $Y = AX^n + B$. When $X = 0$, $Y = 0$;

therefore $B = 0$. Average values for A and n are 13.9 and 0.213 respectively, and the equation becomes $Y = 13.9 X^{0.213}$.

The curve in Fig. 2 is the calculated curve, and the indicated points represent experimental values.

The above result is not in itself capable of any definite interpretation. Presumably the figures for protein represent equilib-

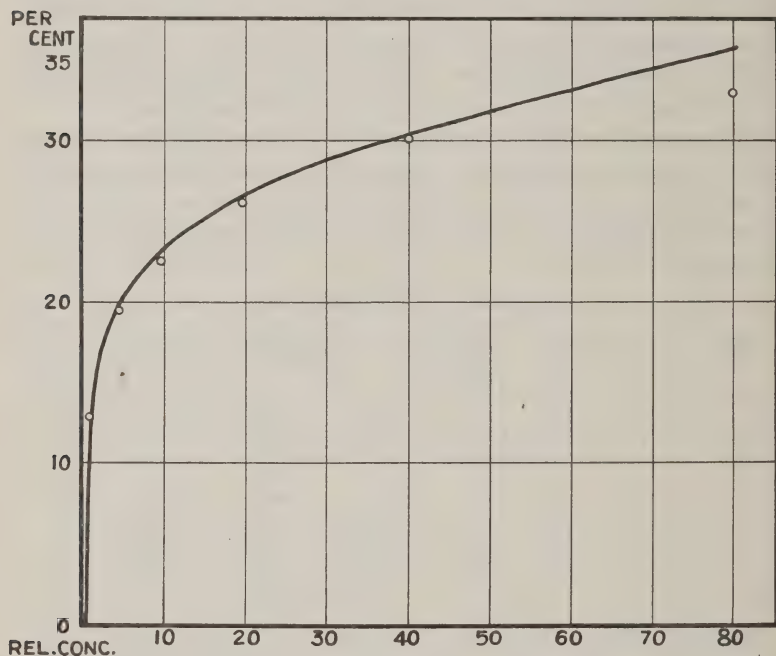


FIG. 2. Relation of pepsin concentration (abscissæ) to equilibrium amount of protein synthesized (ordinates). Curve drawn from empirical equation $Y = 13.9 X^{0.213}$. Points o represent experimental values (data in Table VII).

rium amounts, because the highest concentrations of enzyme employed were relatively enormous, 1 to 8 per cent. Even the lowest, 0.1 per cent, is more than adequate for the complete hydrolysis of 6 per cent albumin in 36 hours, if the pH is maintained at the optimum. To decide the point, the amount of protein synthesized with a low enzyme concentration, 0.5 per cent, was followed over a fairly lengthy period, and the time noted for the

attainment of equilibrium. Two other experiments were also carried out under identical conditions, except that in one case 5 per cent pepsin (Eimer and Amend), and in the other 8 per cent pepsin (Merck) was employed. The results of these experiments are shown in Table VIII.

With 0.5 and 5.0 per cent pepsin, equilibrium is apparently reached in about 36 hours. With greater enzyme concentrations, larger amounts of protein are formed.

If the true equilibrium position of the system can be represented by the final amount synthesized, then we have here an exception to the general law of enzyme action and catalysis, which states that the equilibrium position is not affected by the concentration of the enzyme.

TABLE VIII.

0.5 per cent pepsin (Merck).		5.0 per cent pepsin (Eimer and Amend).		8 per cent pepsin (Merck).	
Time.	Protein N in per cent of total N.	Time.	Protein N in per cent of total N.	Time.	Protein N in per cent of total N.
<i>hrs.</i>		<i>hrs.</i>		<i>hrs.</i>	
6	9.1				
18½	14.1				
24	16.6	24.0	25.0	24.0	33.2
36	18.6				
48	18.6	60.0	27.3		

SUMMARY.

1. The optimum C_H for protein synthesis in peptic digests of egg albumin is at pH 4.0.
2. The hydrogen ion concentration exerts its influence through the ionization of some unidentified di-acid or amphoteric constituent of the digest.
3. Under the conditions in which protein synthesis occurs, there is no appreciable autodestruction of pepsin.
4. The amount of protein formed is partially dependent upon the concentration of pepsin.
5. The quantitative aspect of this relationship precludes the possibility that ionization of the enzyme influences the location of the optimum hydrogen ion concentration.

The authors desire to express their gratitude to Professor Andrew Hunter for his interest and helpful criticism.

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STUDIES ON ENZYME ACTION.

XXIX. COMPARATIVE LIPASE ACTIONS OF DIFFERENT RABBIT TISSUES.

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(Received for publication, November 3, 1924.)

INTRODUCTION.

In the further development of the experimental study of the enzyme actions of tissues and tumors, it was found necessary to determine these actions for a number of tissues of the adult rabbit. This study was carried out, not merely for the purpose of having the data for the comparative actions of the tissues of various animals, but more particularly in connection with the study of the tissues of rabbit embryos, for which the results will be communicated in the near future.

The lipase and protease actions of rat and beef tissues were presented in earlier papers.¹ It was shown, especially for the lipase actions, that each tissue caused definite relative actions on a number of different substrates which when plotted gave a characteristic "picture" of the enzyme actions of that tissue under the fixed conditions used. The absolute enzyme actions found with the different tissues were also significant but, as a rule, not so striking.

EXPERIMENTAL METHODS AND RESULTS.

The experimental methods were practically the same as those described in the earlier papers. The fresh tissue was removed immediately after killing the rabbit by a blow below the atlas,

¹ Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183, 213.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Rabbit Tissues on the Indicated Esters.

Experiment No.	Tissue extracted per cc. mixture tested.	PhOAc	Gl(OAc) ₂	MeOBu	PhCH ₂ OAc	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
Leg muscle extracts.											
	<i>mg.</i>										
E 6 A	17.8	0.42	0.44	0.49	0.14	0.18	0.22	0.37	0.04	0.03	0.15
E 4 G	44.4	0.99	1.02	0.89	0.49	0.39	0.46	0.60	0.16	0.14	0.47
E 3 A	88.9	1.90	1.84	1.46	1.05	0.74	0.94	1.00	0.29	0.15	0.96
Heart muscle extract.											
E 1 B	44.4	1.17	1.14	1.19	0.69	0.49	0.54				
Kidney extracts.											
E 4 H	17.8	1.64	1.59	1.36	0.80	0.69	0.75	1.43	0.45	0.35	0.78
E 3 E	44.4	5.23	4.36	4.30	2.34	2.20	2.43	3.99	1.54	1.27	2.18
Lung extracts.											
E 6 E	17.9	3.20	1.84	2.76	1.04	1.20	1.46	2.40	0.64	0.65	1.12
E 1 C	44.4	4.52	3.14	3.69	1.82	1.72	1.86	3.07	1.12	1.08	1.82
Spleen extract.											
E 3 F	8.9	4.92	3.45	1.74	1.03	0.59	0.62	1.77			
Testes extracts.											
E 3 I	8.9	1.33	0.83	1.23	0.38	0.34	0.41	0.96	0.24	0.21	0.37
E 6 G	17.8	1.89	1.45	1.65	0.93	0.68	0.74	1.57	0.47	0.46	0.96
Brain extracts.											
E 6 F	17.8	0.79	0.46	0.25	0.18	0.14	0.18	0.19	0.04	0.00	0.23
E 3 H	44.4	3.04	1.55	0.93	0.75	0.51	0.59	0.60	0.18	0.04	0.66
Liver extracts.											
E 3 D	8.9	3.96	2.81	3.90	1.74	1.41	1.76	2.99	0.89	0.89	1.64
E 4 C	17.8	4.16	2.98	4.45	1.77	2.37	2.41	3.52	1.31	1.10	1.92
E 1 A	44.4	5.52	5.44	5.85	3.32	3.24	3.59	4.64	1.57	1.41	3.28

ground in a meat chopper, extracted overnight with a definite amount of water, filtered through paper, brought to pH 7.0, and 15 cc. portions taken for the tests with 3.4 milli-equa-

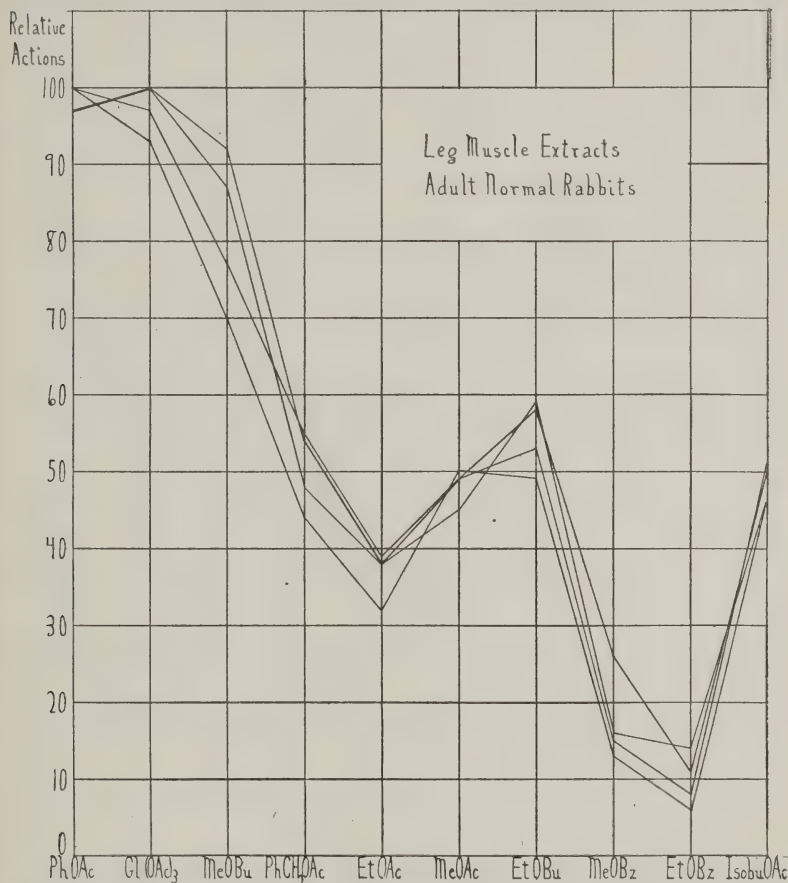


FIG. 1. Leg muscle extracts. The actions on phenyl acetate and glyceryl triacetate are practically the same. Greater actions are shown for the methyl esters than for the ethyl esters. The isomers, ethyl butyrate and isobutyl acetate, also show very much the same actions. Actions on the butyrates are greater than on the corresponding acetates.

lents of ester in each experiment. Results are given for the following esters: phenyl acetate, glyceryl triacetate, methyl butyrate, benzyl acetate, ethyl acetate, methyl acetate, ethyl

butyrate, methyl benzoate, ethyl benzoate, and isobutyl acetate. Toluene was present from the time the tissue extraction was begun. Duplicates and the necessary blanks were run in each experiment. The amounts of enzyme actions were determined

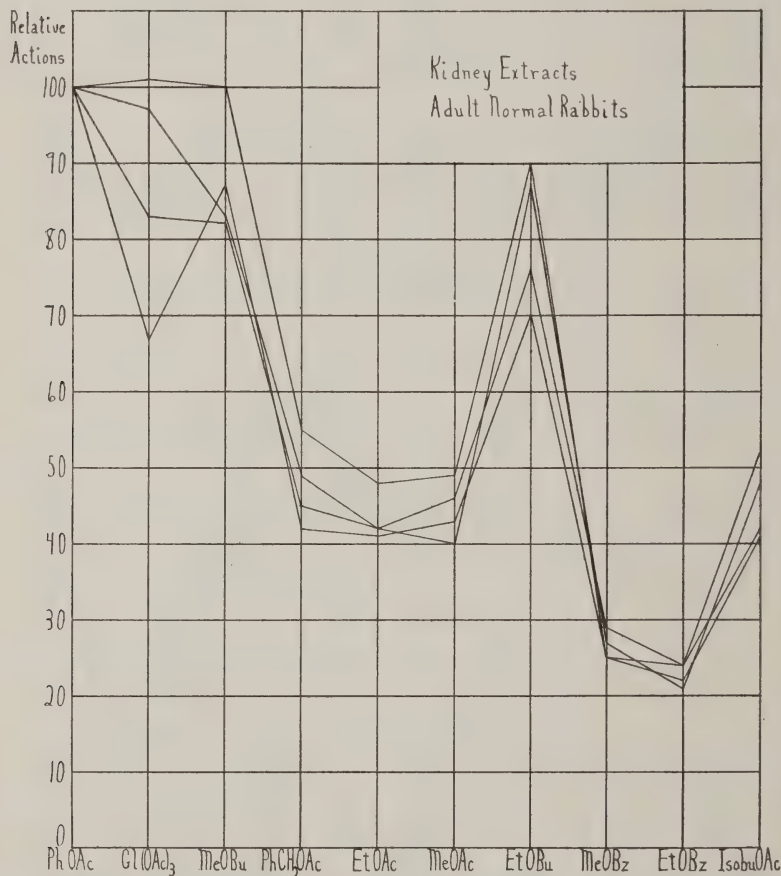


FIG. 2. Kidney extracts. The relative actions on glyceryl triacetate and methyl butyrate show greater variations in comparison with the actions on the other esters than were found in most tests. In general, however, the actions on these two esters may be said to be somewhat smaller than on phenyl acetate. Comparing the methyl and ethyl esters, the actions did not differ much, the methyl ester values being slightly larger. The order of decreasing actions for both methyl and ethyl esters was butyrate, acetate, benzoate.

after incubation at 37°C. for 22 hours by titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator.

The results are presented in Table I and in Figs. 1 to 6. The data given in the table were selected in order to show the

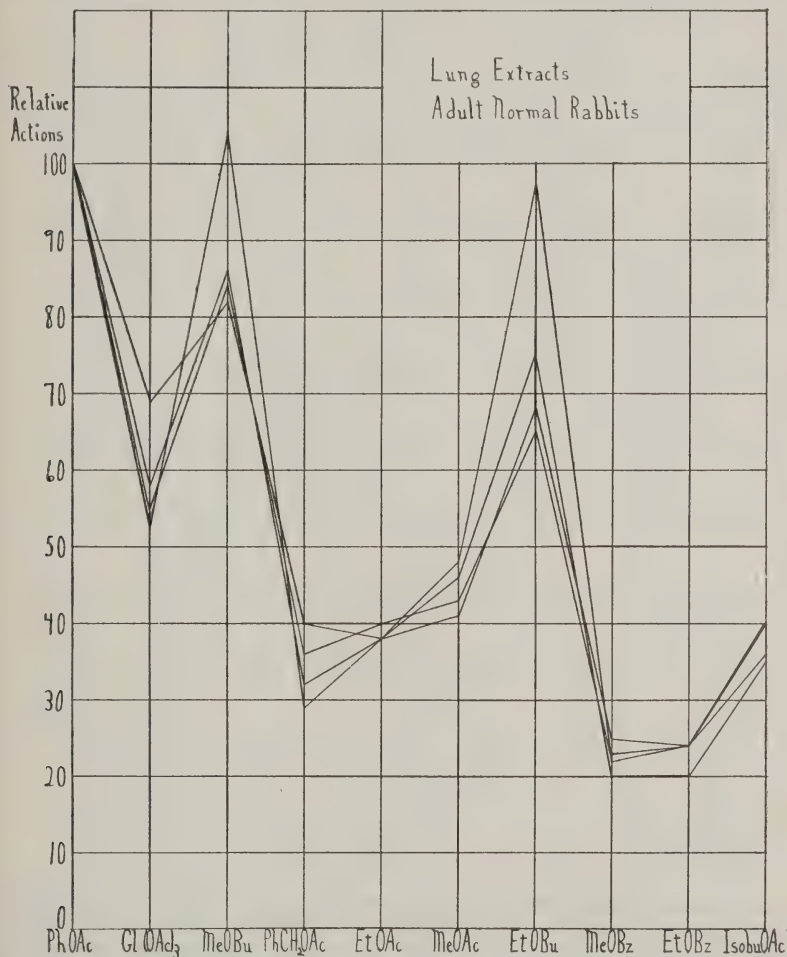


FIG. 3. Lung extracts. The picture of these results is different from those already given. Especially significant is the lower value for glyceryl triacetate as compared with phenyl acetate and the two butyrates. The lower value of ethyl acetate when compared to methyl acetate is also of interest.

magnitudes of the actions of the various tissues at a few different concentrations.

The relative actions of each rabbit tissue on the different

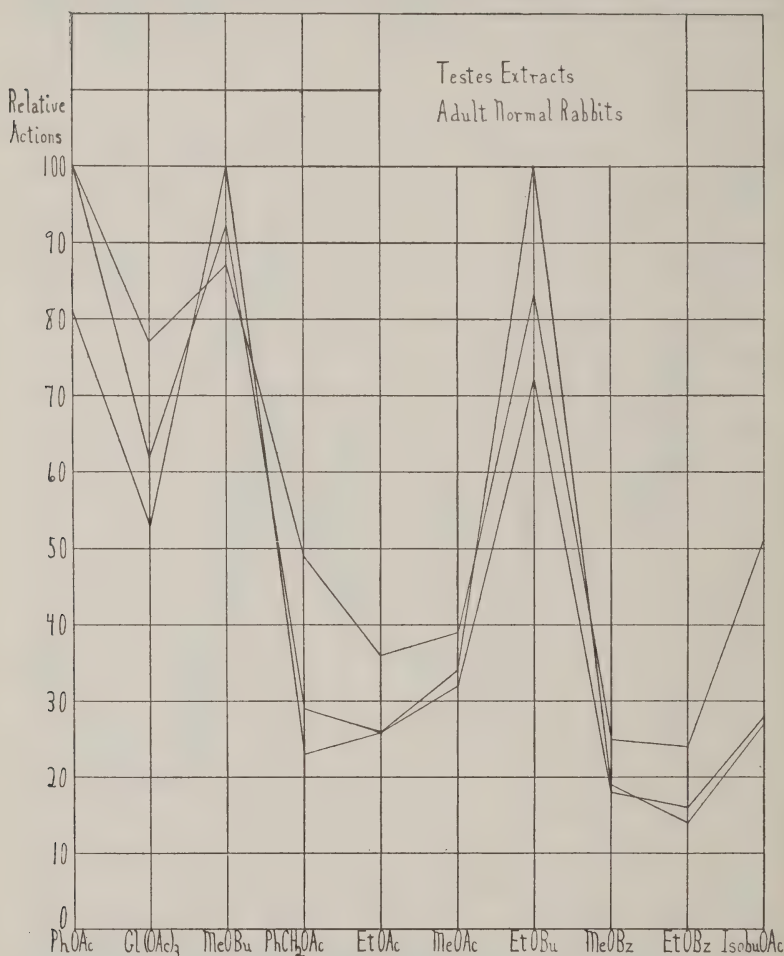


FIG. 4. Testes extracts. Phenyl acetate and glyceryl triacetate showed some irregularities. For the rest, the pictures are similar to those for the lung extracts, except for minor details. It may be noted, however, that the absolute actions for the corresponding concentrations are considerably smaller (half as large at times) for the testes extracts in comparison with those of the lung extracts.

esters are plotted in Figs. 1 to 6. The order in which the esters are arranged is the same as that used in the previous papers.

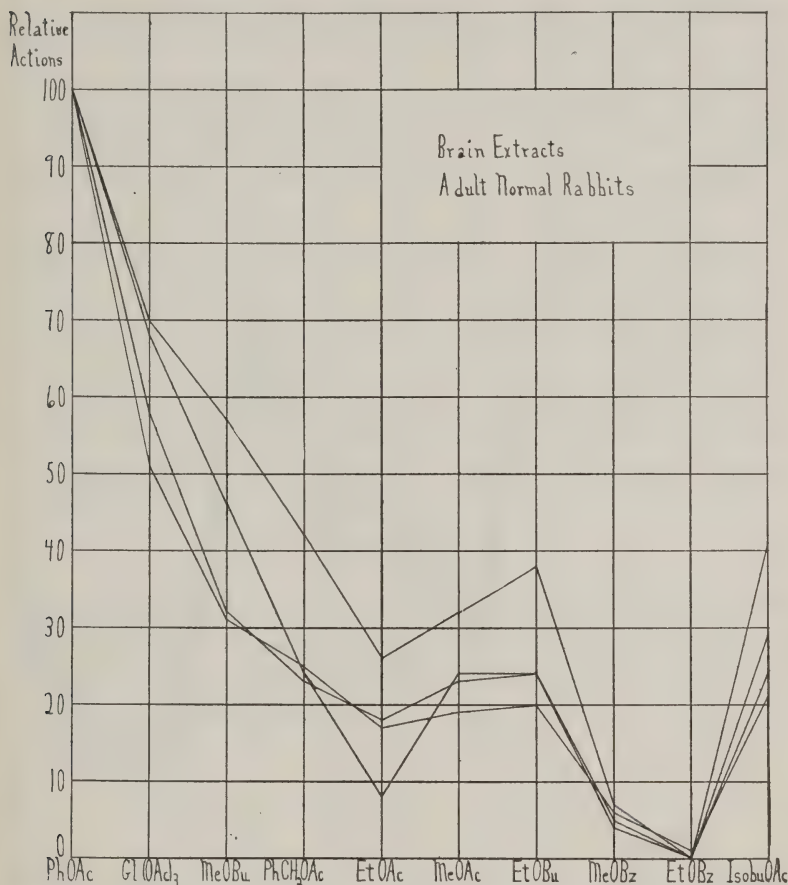


FIG. 5. Brain extracts. The picture of these results is different again from those of the other tissues. Especially noteworthy are the comparatively small actions of the two butyrates, the same actions on the isomers, ethyl butyrate and isobutyl acetate, and the very small actions on the benzoates.

No curves are given for the rabbit spleen or heart. Several incomplete series of actions were obtained with each, insufficient for satisfactory plots. Some of the absolute actions obtained

with these are, however, shown in Table I. A more complete study of the rabbit skin actions will be presented in another connection.

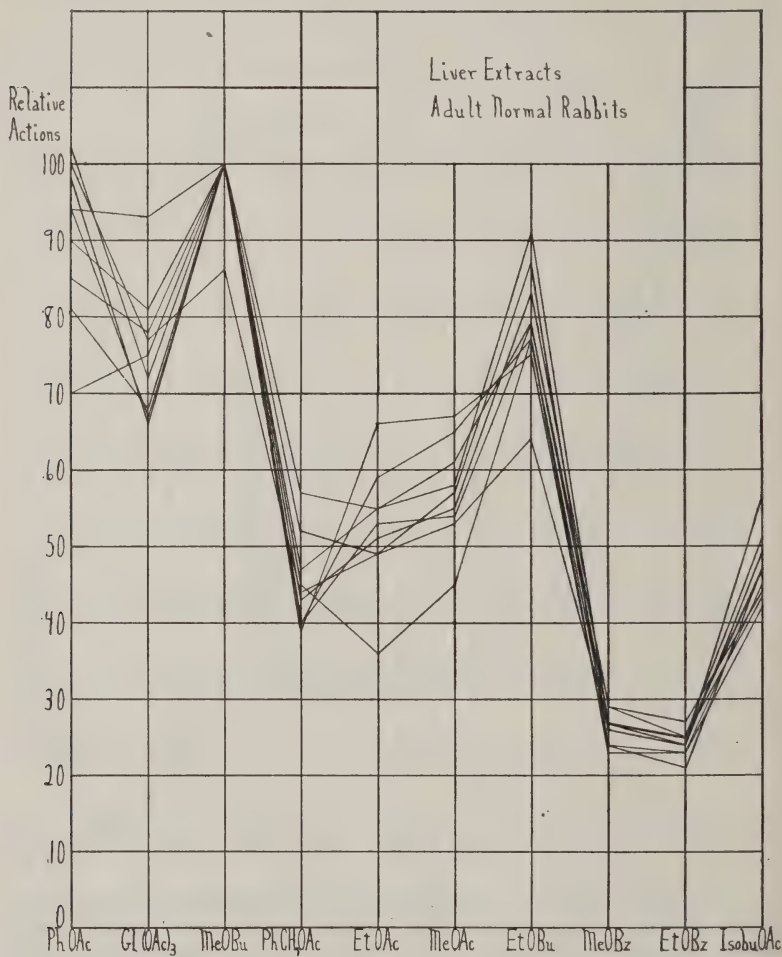


FIG. 6. Liver extracts. The first important feature in these actions is their large general value. Marked actions are shown on all the esters studied. This is apparent also in the absolute actions given in Table I. Irregularities are seen with certain esters in some of the series, but the general trend with the large number of results available is apparent. The high relative values of the butyrates may be noted as well as the small differences between the methyl and ethyl esters of corresponding acids.

The curves show fairly characteristic relations for the lipase actions of the different rabbit tissue extracts. The absolute actions on the different esters for the same concentrations of material extracted, some of the values being given in Table I, may be stated roughly to decrease in the following order: liver, lungs, testes, kidney, brain, leg muscle. The isolated results with spleen show an order of magnitude corresponding to that of liver (although the pictures appear to differ strikingly), while those with heart muscle are similar to the leg muscle results, although somewhat larger. The order which is given for the absolute actions of the different tissues is only a rough approximation. For certain esters a different order would be obtained, but the order given represents the general view of the actions. Similar relations were pointed out for results with rat and beef tissues communicated previously. The conclusion presented there applies to the results given here; namely, that the relative actions of the various tissues on the different esters show the most characteristic relations, but that for a more complete understanding of the results, the absolute actions must also be considered.

SUMMARY.

The ester-hydrolyzing actions of a number of tissues of the adult rabbit on ten esters were determined. The results are presented in the form of curves for the relative actions and in a table for the absolute actions.

STUDIES ON ENZYME ACTION.

XXX. A COMPARATIVE STUDY OF THE CHARACTERISTIC LIPASE ACTIONS OF THE TISSUES OF DIFFERENT ANIMALS AND OF SOME HUMAN TISSUES.

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(Received for publication, November 3, 1924.)

INTRODUCTION.

The characteristic lipase actions or "pictures" of a number of tissues, each from several different animals, are compared in this paper. Such "pictures" of the enzyme actions for a number of tissues were obtained under definite and standard conditions for the rat (1), rabbit (2), and beef (3). The averages obtained in each case from a number of the individual results will be used here. It was possible to obtain at various times certain human tissues from autopsies. A limited number of results obtained with such tissues are at hand, and it seemed worth while to include them here. Much stress cannot be placed upon these last results. While they may, and in most of the cases probably do, represent characteristic actions for the tissues in question, at the same time it does not appear that these tissues represent the same state of "normality" or "average condition" that is represented by tissues of freshly killed healthy animals. The human material was obtained at intervals through the courtesy of the Staff of The Roosevelt Hospital and of Dr. W. C. Johnson of the Department of Pathology, College of Physicians and Surgeons, Columbia University. The averages only, of the human results will be presented. In view of their element of uncertainty, the separate results may be omitted, and any significant facts pertaining to the absolute actions found, stated.

Presentation of Results.

The results will be presented entirely in the form of curves (Figs. 1 to 9). Each figure gives the average results of the extract

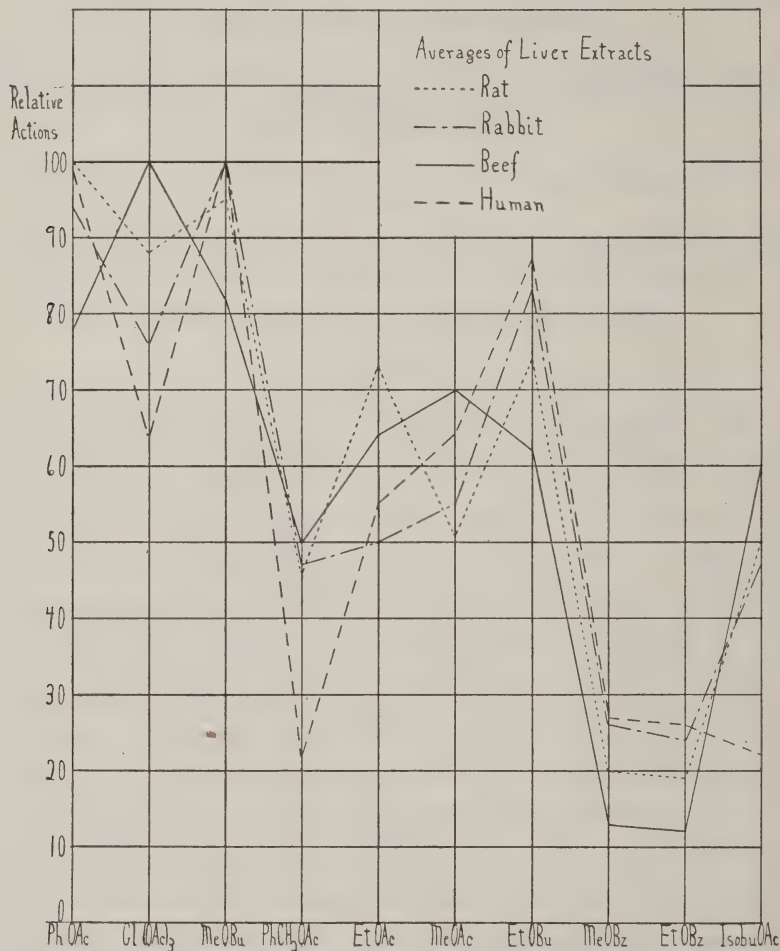


FIG. 1. Liver: rat, rabbit, beef, and human. The rat, rabbit, and human livers show a general similarity; the beef liver being quite different. The low relative actions of the human liver on benzyl acetate and isobutyl acetate are to be noted, as well as the fact that the actions of all on phenyl acetate and methyl butyrate are of the same order of magnitude, and on ethyl butyrate, not much less. Except for the beef, the butyrates show higher values than the corresponding acetates.

of a definite tissue from a number of different animals. The esters are indicated at equidistant intervals along the abscissa axis; the relative amounts of the esters hydrolyzed given as percentages of the greatest hydrolyzing action found for that animal tissue are

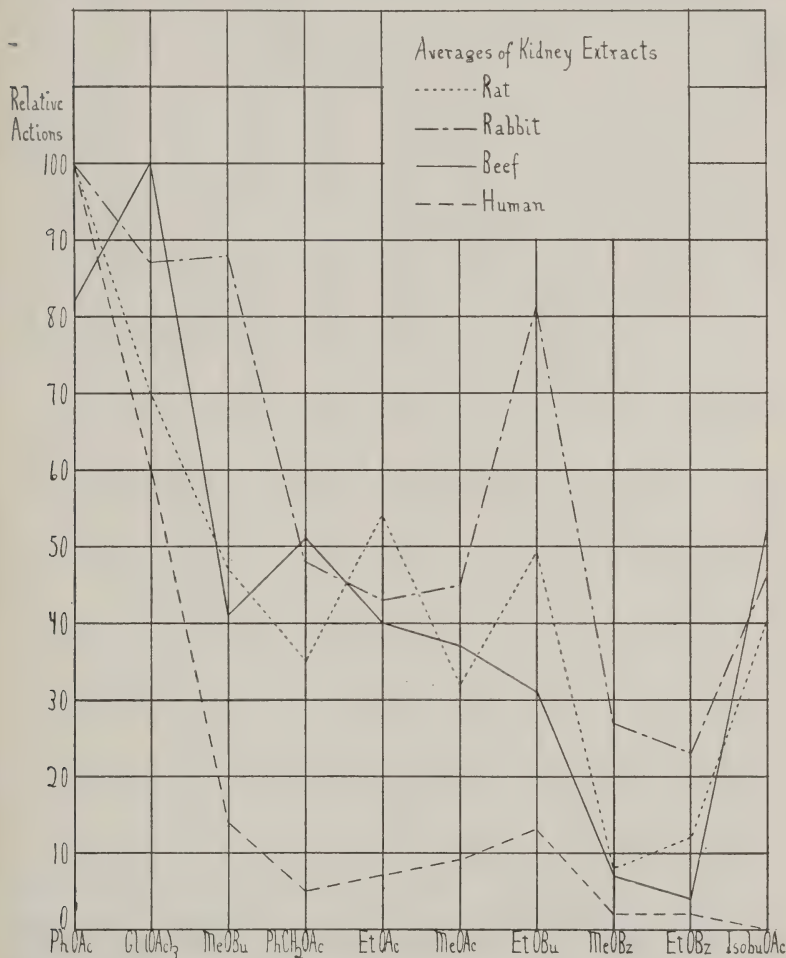


FIG. 2. Kidney: rat, rabbit, beef, and human. The four curves show markedly different types of actions. The relatively high actions on the butyrates shown by the rabbit extracts, and the low actions on all esters except phenyl acetate and glyceryl triacetate by the human, may be specially noted.

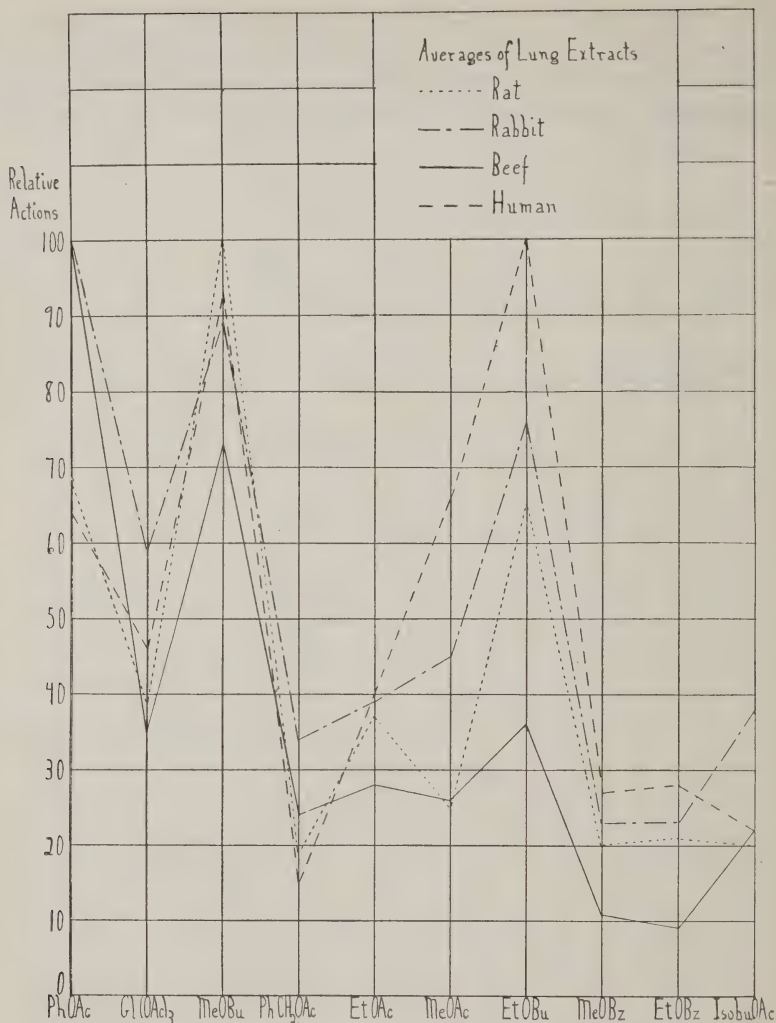


FIG. 3. Lung: rat, rabbit, beef, and human. There is a general similarity in the four pictures. The high values on methyl butyrate of all the extracts, and of the human and rabbit extracts on ethyl butyrate, may be noted. Comparing the two butyrates with each other, the ratio methyl: ethyl increases in the order; human, rabbit, rat, beef to a value of 2:1. The actions on the butyrates are greater in every case than on the corresponding acetates, while for the two isomers, ethyl butyrate and isobutyl acetate, the values throughout are greater for the former.

shown on the ordinate axis. The description of the results will be given with the different figures.

The figures which have been presented illustrate the character of the results which were obtained. Limiting the discussion for the

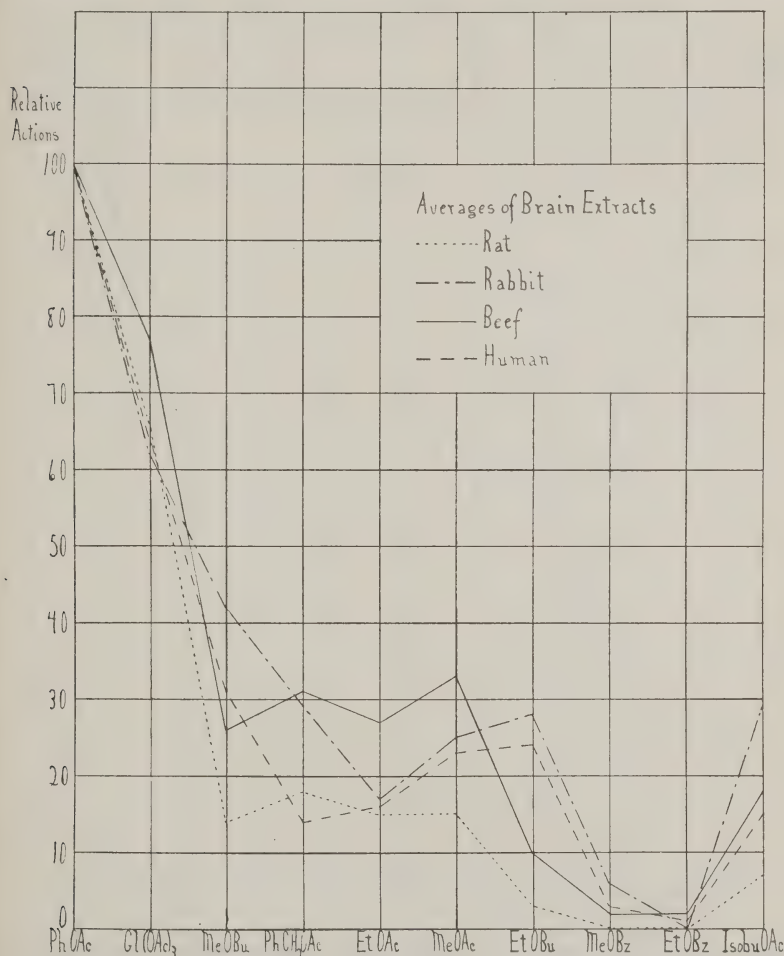


FIG. 4. Brain: rat, rabbit, beef, and human. There is marked similarity in the actions of the different extracts, more so than with any other tissue studied. The differences which can be observed are of minor character and include the smaller relative actions of rat and beef on ethyl butyrate, etc.

present to the curves of relative actions, it is seen that for some tissues the curves are practically identical for all of the animals studied (for example, the brain); for other tissues, the curves may

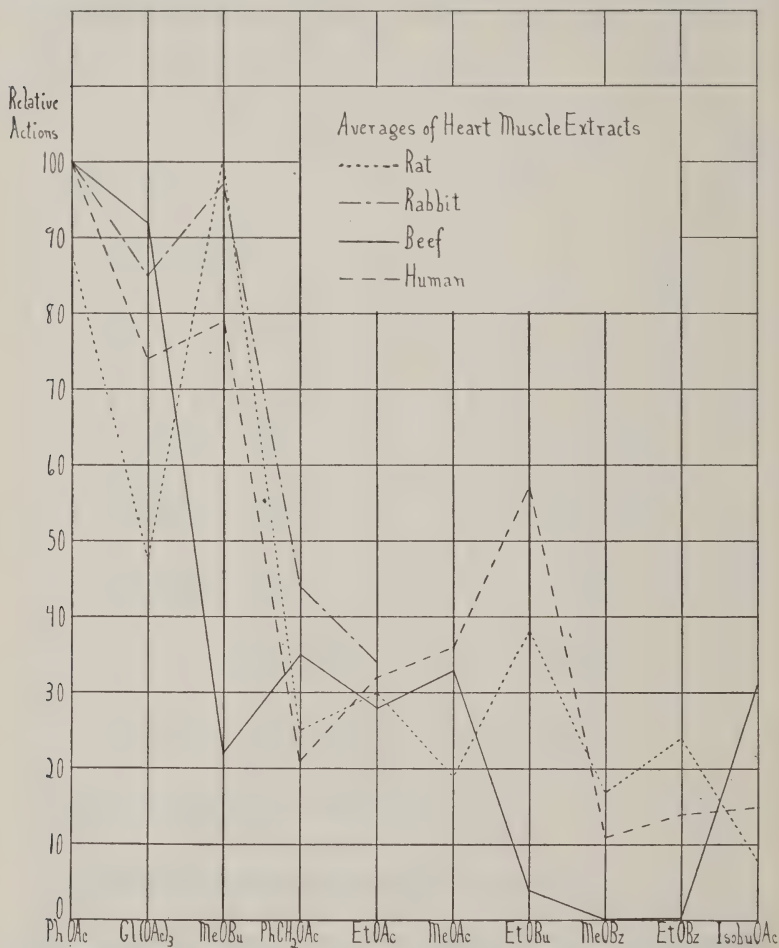


FIG. 5. Heart muscle: rat, rabbit, beef, and human. Definite differences are shown in these curves; the high values of rat and rabbit on methyl butyrate as against the low value of beef, the low glyceryl triacetate action of rat, the low ethyl butyrate value of rat in comparison with the methyl butyrate, etc. There is no marked similarity between any two of the sets of averages.

be similar for some animals and different for others (for example, the spleen and the liver); while for others again there are differ-

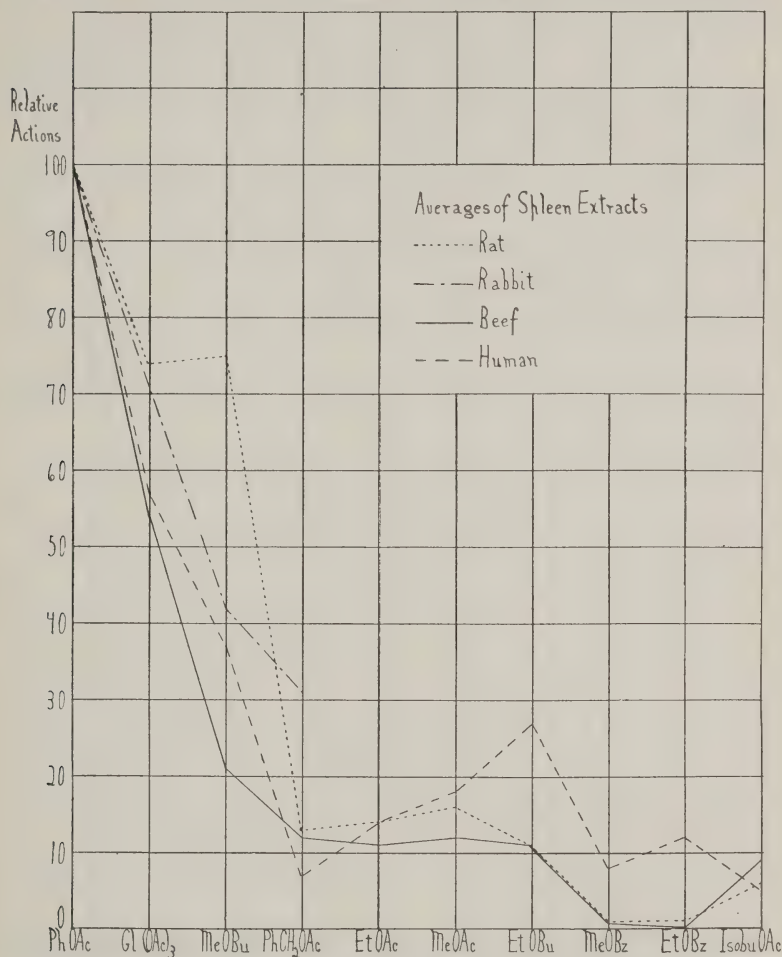


FIG. 6. Spleen: rat, rabbit, beef, and human. Except for the high methyl butyrate value of the rat, these curves are very much the same. With the same exception, they are similar to the curves for the brain extracts of the different animals except that the relative values for the esters following the first three are of smaller magnitude even than those for the brain extracts. In view of their small values, any differences or similarities which may appear with these relative actions may, for the present, be discounted.

ences for all the animals studied (for example, the heart muscle and the kidney). It is clear, of course, that the results presented

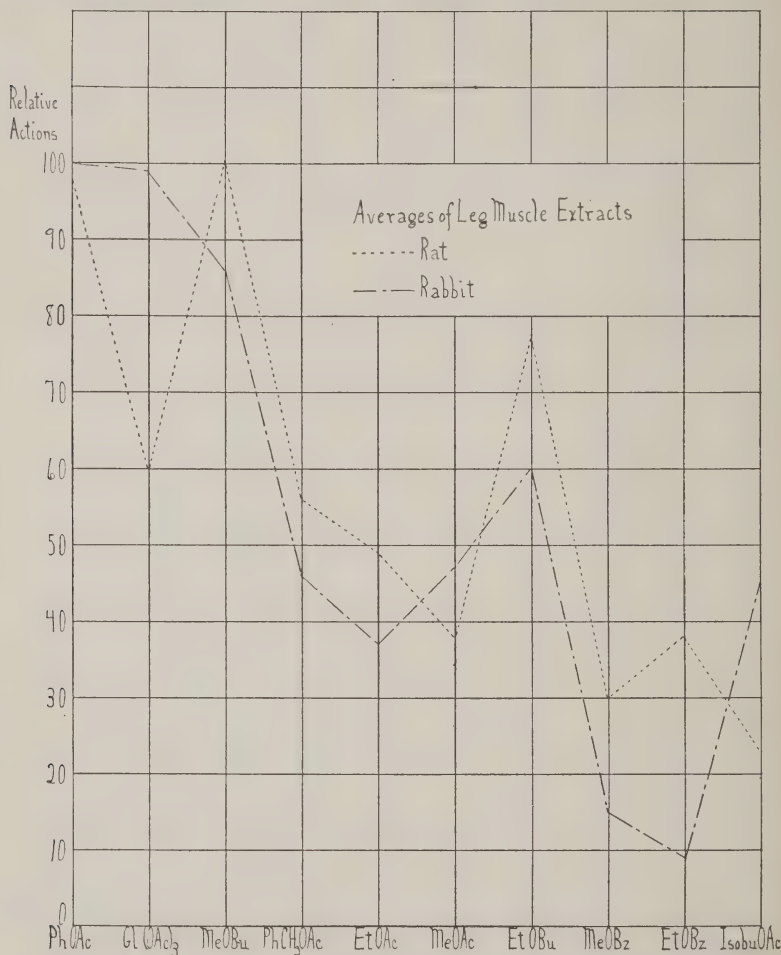


FIG. 7. Leg muscle: rat and rabbit. There is a certain similarity in these two curves as shown by the relatively high butyrate values and a difference as shown, for example, by the high glyceryl triacetate and isobutyl acetate values of the rabbit as against the lower values of the rat. Other relations are obvious if pairs of esters are considered, as ethyl acetate larger than methyl acetate for the rat and the reverse for the rabbit, etc.

are limited both in the number of different animals which were studied and in the number of different esters which were used as substrates. Further extension of this study in the two directions unquestionably would show additional differences as well as similarities in the curves of relative actions.

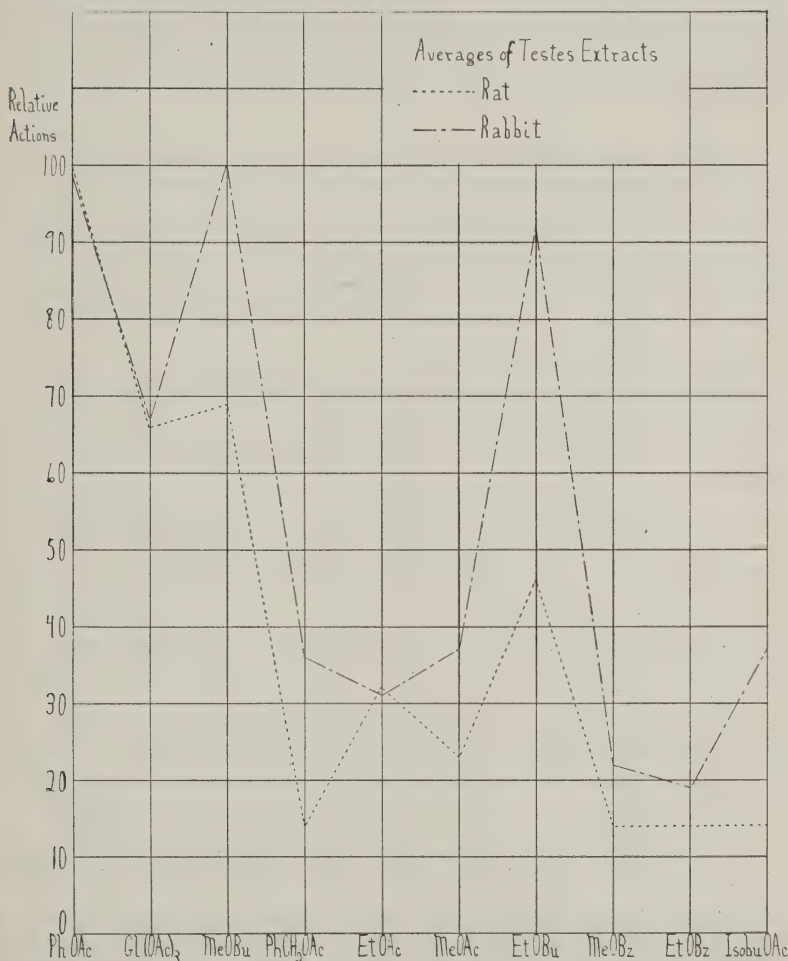


FIG. 8. Testes: rat and rabbit. The most striking differences in these curves are the high values of methyl butyrate and ethyl butyrate for the rabbit. Differences of minor character include the small actions of the rat on benzyl acetate, methyl acetate, and isobutyl acetate.

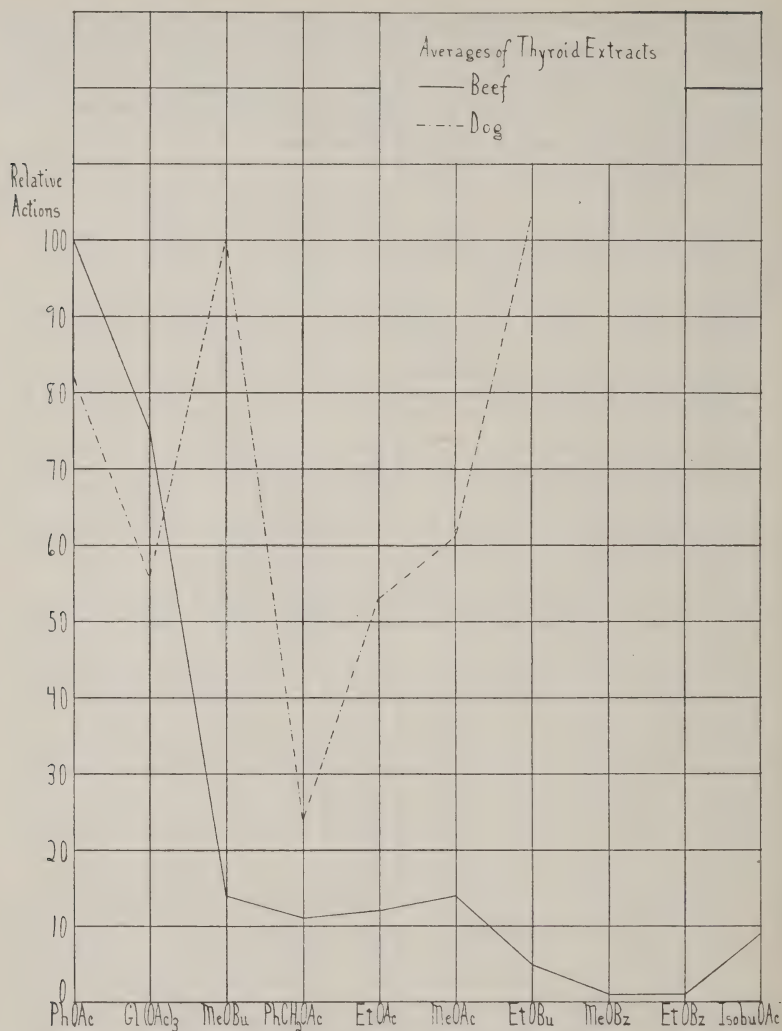


FIG. 9. Thyroid: beef and dog. These results have been included as considerable of this material was studied. The beef thyroid showed results very much like the beef spleen, while the curve for the dog thyroid is entirely different. The general high level of the actions following the first two esters in comparison with the beef, is striking, as well as the high butyrate actions and low benzyl acetate value.

The curves for the relative actions of the tissues of different animals, while probably the more significant, do not tell the whole story. For a more satisfactory understanding of the relations, it is necessary to include the absolute enzyme actions of the various tissue extracts under conditions which are comparable as far as possible. Two methods of comparison may be indicated; namely, the absolute actions on the esters of the same tissue of different animals, and the absolute actions of different tissues of the same animals.

The comparison of the absolute actions of the extracts of tissues from various sources will depend to a certain extent upon the ester used, since the "pictures" of the relative actions differ frequently. The conclusions, based as far as possible upon comparable concentrations, may be given for the same tissue from the different animals, as follows:

Liver.—Rat larger than rabbit for all esters; rat larger than beef for phenyl acetate and the two butyrates; beef larger than rat for the remaining esters; beef larger than rabbit practically throughout; human as small as, or smaller than the rest.

Kidney.—Rat largest by far, rabbit next, beef next. Human possibly slightly larger than rabbit.

Lung.—Rat largest; beef and rabbit, some esters larger for one, some for the other; human in general, smallest.

Brain.—All actions small; order of decreasing values; rat, rabbit, beef, and human.

Heart Muscle.—Rat apparently largest; rabbit and human, not much different; beef, small especially after phenyl acetate and glyceryl triacetate.

Spleen.—Rat values larger than rabbit for phenyl acetate, glyceryl triacetate, and methyl butyrate, smaller for benzyl acetate and ethyl butyrate. Human values smaller than rat and rabbit values, but larger than beef.

Leg Muscle.—Absolute actions small both for rat and rabbit, perhaps somewhat larger for former.

Testes.—Rabbit values much smaller than rat.

Thyroid.—Phenyl acetate action the same for beef and dog; glyceryl triacetate action, dog slightly larger; dog much larger for remaining esters.

Although it is obviously impossible to state definite rules as to the magnitudes of the actions for the same tissue in different animals because of the differences in relative actions on the various

esters, a general conclusion may be given. As a rule, the rat tissues are the most active, followed by rabbit, beef, and human tissues in the order named. This applies to the special experimental conditions used and only in the most general terms.

As for the absolute actions of the various tissues of the different animals, the only general statements which may be given point to the facts that the brain and muscle tissues show the smallest actions.

Another type of relation, which may prove to be of considerable importance, may be indicated. In comparing the pictures of actions, it is seen that, of all the tissues studied, only the brain of the various animals gave essentially the same type of action for all. Further, it has been found that the Flexner-Jobling rat carcinoma gave a definite "picture" for its ester-hydrolyzing enzyme actions (1) and that a number of tumors of human origin gave essentially the same picture (4). The pictures of the brain actions and these tumor actions are practically the same, while the absolute actions are also of the same order of magnitude. It may be pointed out, also, that these absolute actions are relatively small, except for phenyl acetate and glyceryl triacetate, especially in comparison with the absolute actions of most of the other tissues. Also, all tumors or abnormal growths do not give the same picture. For example, the Twort and Bashford 63 mouse carcinomas showed definite and characteristic differences in comparison with the Flexner-Jobling rat carcinoma and human tumor actions (5). At the same time, the similarity in these enzyme actions of the brains of all animals studied and the enzyme actions of a number of tumors, may have a deeper significance than is apparent at first sight.

It was found that several additional tissues gave "pictures" or types of actions similar to the Flexner-Jobling rat carcinoma type. These include beef and human spleens, beef heart, beef thyroid, and human kidney. The absolute actions of the spleens were much greater than those of the brains for corresponding concentrations, so that this would serve to distinguish and group them. The beef heart actions on the other hand were small, greater than beef brain apparently, but smaller than rabbit brain. The beef thyroid actions were very small for all except the first two esters, and comparatively greater for the glyceryl triacetate than with

the other tissues showing apparently similar types of actions. As for the human kidney, the actions were greater than the brain actions, but in view of the uncertainty involved in all the tissues of human origin which were studied, much significance cannot be ascribed to these results at the present time.

The general conclusion which may be derived from the results presented in this paper points to the fact that the study of the type of action or "picture" of the ester-hydrolyzing actions of a given tissue of an animal together with the absolute actions, both under standard conditions, may be taken to be characteristic in most cases. A number of similarities of actions were observed and indicated. The discussion of such similarities as well as of the differences might be continued indefinitely. From the chemical point of view, however, enough has been said. It is possible that more light will be thrown upon the various relations by considering the physiological aspects of the various tissues in connection with the enzyme actions which have been described.

SUMMARY.

The average "pictures" of the lipase actions on ten esters of nine tissues of the rat, rabbit, beef, human, and dog (one tissue) are compared in a series of figures. Taken in connection with the absolute actions under comparable conditions, definite similarities and differences are pointed out.

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THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

II. HYDROLYSIS WITH ENZYME (EREPSIN).

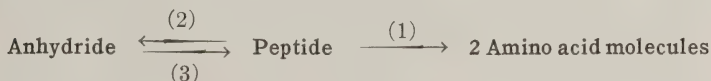
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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 1, 1924.)

The first paper¹ described the hydrolysis of peptides with hydrochloric acid. Molar solutions of the following four peptides were hydrolyzed with a known excess of H ion at 100°C.: glycyl-glycine, sarcosyl-glycine, glycyl-sarcosine, and sarcosyl-sarcosine. These will be referred to by the symbols: GG, SG, GS, and SS, respectively.

It was found that three reactions occurred simultaneously: (1) the peptide split into amino acids (a simple bimolecular reaction, when one equivalent of active H ions is present at the start), (2) the peptide formed a ring compound (anhydride), and (3) the anhydride opened up, forming peptide.



The synthesis of peptide from amino acid was negligible.

If we compare the four peptides with regard to reaction (1) we find that the resistance to hydrolysis is qualitatively proportional to the product of the dissociation constants of the groups involved in the linkage. Similarly, the resultant of the two reactions (2) and (3) indicates that the stability of the anhydride bond is roughly proportional to the products of the constants of the groups involved.

¹ Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, 1924, lxi, 445. In Table II of that article, Column 5 should be corrected to read: 1,000 || 250 || 17.5 || 5.1.

Table I gives the values of the velocity constants as predicted and as found in acid hydrolysis. In addition, the values given in Columns 5, 6, and 7 are calculated on the assumption that the bond is proportional to the square of the product of the dissociation constants. The agreement with the values found (Columns 8, 9, 10) is seen to be more nearly quantitative on this basis. The values for GG, SG, and SS are quantitative, within experimental error. GS forms an exception.

In this paper it will be shown that the same laws hold with enzyme hydrolysis as with acid hydrolysis. The same four peptides when hydrolyzed with erepsin at 40°C. appear to simultaneously hydrolyze and form anhydride at rates cor-

TABLE I.
Relative Values of the Velocity Constants.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	Predicted from $K_a K_b$.			Predicted from $(K_a K_b)^2$.			Found in acid hydrolysis.		
Constant.	k_1	k_2	$\frac{k_2}{k_1}$	k_1	k_2	$\frac{k_2}{k_1}$	k_1	k_2	$\frac{k_2}{k_1}$
Reaction.	(1) Split- ting.	(2) Anhy- dride.	(2) (1)	(1)	(2)	(2) (1)	(1)	(2)	(2) (1)
GG.....	1.0	1.0	1.0	1.0	0.053	0.018	1.0	0.10	0.04
SG.....	0.79	2.9	3.7	0.63	0.437	0.24	0.64	0.40	0.28
GS.....	0.43	5.7	13.2	0.19	1.74	3.1	0.57	5.7	4.4
SS.....	0.34	20	59	0.116	20	59	0.15	20	59

responding to the values of $(K_a K_b)^2$ (*i.e.*, the square of the product of the dissociation constants of the groups involved in the respective linkages). Except for the fact that reaction (1) is here monomolecular, the two reactions (1) and (2) appear to occur exactly as in acid hydrolysis. Reaction (3) is negligible in erepsin hydrolysis, as is shown in Experiment V, Table VI.²

² This is probably due to two factors: first, the production of anhydride (2) in acid hydrolysis raised the H ion concentration, thus *accelerating* reaction (3) and producing an equilibrium dependent upon the amount of acid present. With enzyme hydrolysis the formation of anhydride *inhibits* reaction (3) due to the removal of the active (neutral) molecules. Furthermore, the point of linkage of the rigid anhydride molecule may be relatively

If, according to our hypothesis, we assume that any dipeptide in the presence of erepsin gives x mols of amino acid and y mols of anhydride in time t , and we start with a mols of peptide; there remain, at time t , $a - x - y$ mols of peptide. If k_1 and k_2 are the constants for reactions (1) and (2), respectively:

$$(1) \quad \frac{dx}{dt} = k_1 (a - x - y) \quad \text{and}$$

$$(2) \quad \frac{dy}{dt} = k_2 (a - x - y)$$

Hence

$$(3) \quad \begin{aligned} \frac{d(x+y)}{dt} &= (k_1 + k_2) (a - x - y) \\ &= k(a - x - y) \quad \text{where } k = k_1 + k_2 \end{aligned}$$

Therefore

$$(4) \quad kt = \ln \frac{a}{a - (x + y)}$$

But we cannot measure $(x + y)$; what we observe is $(x - y)$.

Therefore, if we place $Z = \frac{x - y}{x + y} = \frac{k_1 - k_2}{k_1 + k_2}$ (from equations (1) and (4)); we get

$$(5) \quad kt = \ln \frac{aZ}{aZ - X} = \ln \frac{a'}{a' - X}$$

where

$$X = x - y$$

and

$$a' = aZ = a \frac{x - y}{x + y} = a \frac{k_1 - k_2}{k_1 + k_2}$$

more accessible to the small sized H ion than to the more bulky enzyme molecule. In Experiment V erepsin split GG anhydride very slowly if at all.

If we assume that the neutral molecule of peptide is the active one, as the results of Dernby³ seem to indicate, we should write:

$$(6) \quad kt = \ln \frac{(a - B)Z}{(a - B)Z - X} = \ln \frac{A'}{A' - X}$$

where B = equivalents of base (NaOH) present,

$$(7) \quad \text{and } A' = (a - B)Z = (a - B) \frac{x - y}{x + y} = (a - B) \frac{k_1 - k_2}{k_1 + k_2}$$

TABLE II.

Summary of Velocity Constants with Erepsin.

The constants (k) in Column 6 correspond to the empirical values of A and give curves which correspond throughout with the experimental data; k' is the initial velocity where A' equals the concentration of neutral peptide molecules.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Experiment No.	Peptide.	B Concentration of base.	E Enzyme.	A (found).	$k \times 100$ (found) (equation (8)).	"Corrected" $k' \times 100$ (equation (9)).
I	GG	0.40	cc. 5.0	0.74	9.1	12.1
	SS	0.40	5.0	-0.55	55*	
II	GG	0.40	5.0	0.72	7.6	9.7
	SG	0.40	5.0	0.28	3.0	
	GS	0.40	5.0	(?)	(?)*	
III	GG	0.20	5.0	0.80	8.9	9.2
	SG	0.20	5.0	0.39	5.0	
	GS	0.20	5.0	(?)	(?)*	
	SS	0.20	5.0	-0.80	20*	
IV	GG	0.30	0.2	0.50	2.0	1.4
	GG	0.30	1.0	0.60	5.1	4.3
	GG	0.30	3.0	0.84	6.5	7.9
	GG	0.60	3.0	1.0	2.3	6.3
	GG	0.05	3.0	1.0	4.4	4.4

* The asterisk indicates that k_2 is greater than k_1 (i.e. anhydride formation predominates over splitting).

³ Dernby, K. G., Inaugural dissertation, Hochschule zu Stockholm, 1917.

All the hydrolyses on GG, SG, and SS with erepsin (see Figs. 1 to 3 and Table IV) followed a reaction of the general form of equation (6); namely,

$$(8) \quad kt = \ln \frac{A}{A - X}$$

where A (Column 5, Table II) was determined by "trial and error" until a value was found which gave a constant value of k (equation (8)). (The same result may be obtained from two points on the curve such that $t_2 = 2t_1$. By substituting X (observed) and eliminating k , one can solve for A .)

To follow the progress of the hydrolysis of methylated peptides, it is necessary to titrate with NaOH (using tropeolin O as indicator). To do this the concentration must be so high (molar) that it is not possible to buffer the solutions. The concentration of neutral molecules, however, has the value $(a - B - x - y)$ throughout the reaction until the pH is raised to the buffer range of the amino acids produced. In that range the hydrolysis is faster than that required by equation (6). Consequently the values of A observed (particularly for GG) are not quantitatively equal to $(a - B) \frac{k_1 - k_2}{k_1 + k_2} = (a - B) Z$

The "corrected" constant k' for GG in Column 7, Table II, has been calculated from the equation:

$$(9) \quad \frac{k'}{k} = \frac{\log \frac{A'}{A' - X}}{\log \frac{A}{A - X}}$$

where A' is the calculated value of $(a - B) Z$, from the values of Z calculated in the next section. X is given a small value (e.g. 0.10).

k' represents a curve having the same initial velocity but approaching the limit A' (calculated) rather than the limit A (observed). It is the constant which should represent $k_1 + k_2$ if the buffer effect of the amino acid did not interfere. These values of k' have been used in Table III for the calculation of $k_1 + k_2$ for the other peptides.

Calculation of $R = \frac{k_2}{k_1}$, of Z , and of k .

We may calculate the value of R for a peptide from the formulas:

$$(10) \quad Z = \frac{A}{a - B} \quad \text{and}$$

$$(11) \quad R = \frac{1 - Z}{1 + Z} = \frac{k_2}{k_1}$$

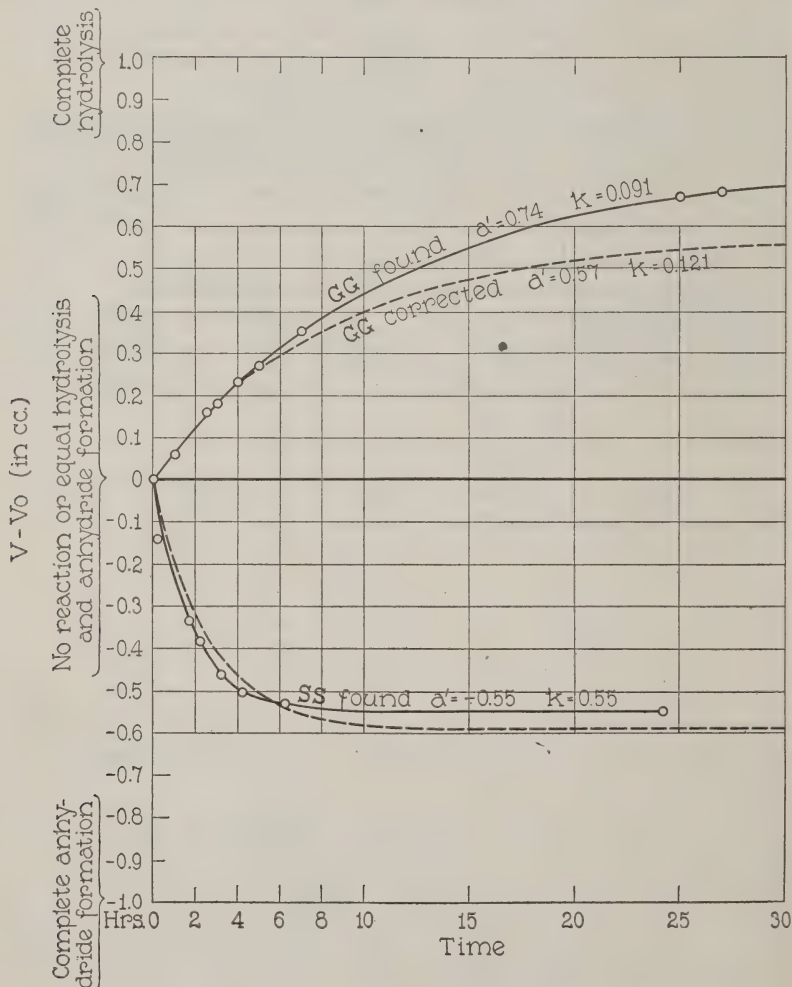


FIG. 1.

if we assume equal steric hindrance in the splitting and in the anhydride formation.

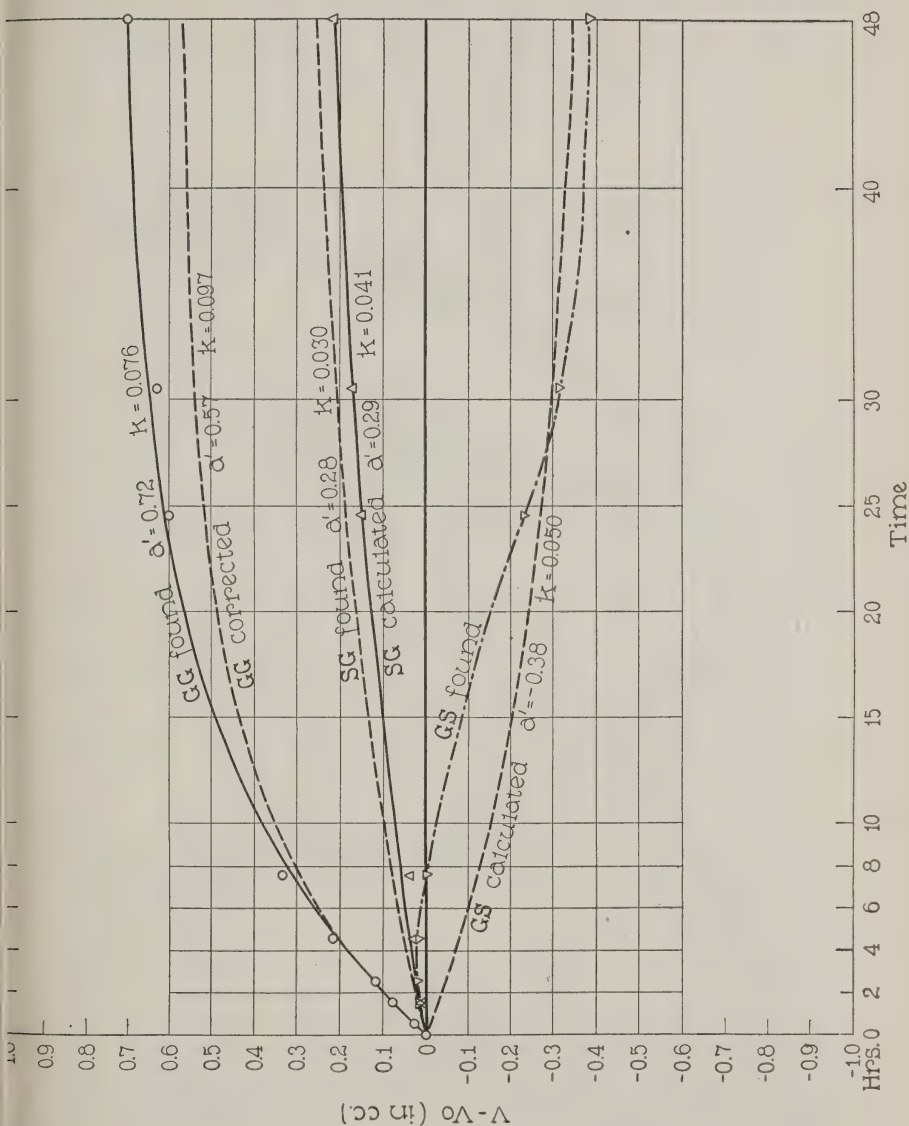


FIG. 2.

Peptide.	Experiment No.	A	Z	R	R_{gg}
SG	II	0.28	0.47	0.36	0.027
GS	II	(-0.38)	(-0.63)	(4.4)	(0.026)
SG	III	0.39	0.49	0.35	0.026

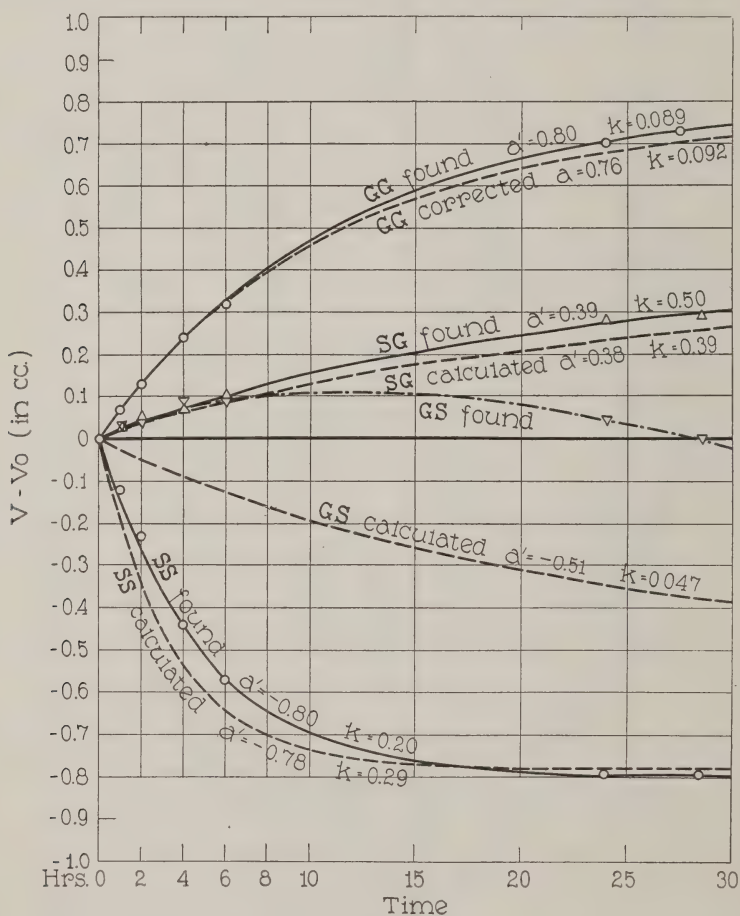


FIG. 3.

The values in the last column are calculated from the relative values of R in Column 2, Table III, which are calculated from the dissociation constants.

If we assume that $R_{GG} = 0.026$, we can calculate R for the other peptides (see Column 3) from the relative values in Column 2. Knowing R for any peptide we may calculate Z from the equation:

$$(12) \quad Z = \frac{1 - R}{1 + R}$$

TABLE III.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Peptide.	$R = \frac{k_2}{k_1}$		Z	$A' = Z(a - B)$ (calculated). A (observed) in bold faced type.		$k = k_1 + k_2$				
	Relative.	Calculated ($k_{GG} = 0.026$).		Experiments I and II.	Experiment III.	Relative. Calculated.		Calculated and found.		
						Uncorrected.	Corrected.	Experiment I.	Experiment II.	Experiment III.
GG	1	0.026	0.95	0.57 0.74 0.72	0.76 0.80	1.0	1.0	0.121 0.091	0.097 0.076	0.092 0.089
SG	13.2	0.36	0.48	0.29 0.28	0.38 0.39	0.84	0.42		0.041 0.030	0.039 0.050
GS	173	4.5	-0.64	-0.38 -0.38	-0.51 (?)	1.02*	0.51*		0.050* (?)	0.047* (?)
SS	3,280	85.0	-0.98	-0.59 -0.55	-0.78 -0.80	9.7*	3.2*	0.39* 0.50		0.29* 0.20

The upper numbers in each square in *Columns 5, 6, 9, 10, and 11* are calculated. The lower numbers (bold faced type) are those found. In the calculation of A' steric hindrance is neglected (on the assumption that it is equal for k'_1 and for k_2). *Column 7* gives the relative values of k calculated from the square of the products of the dissociation constants. In *Column 8* these are corrected for steric hindrance by dividing by the empirical values 1, 2, 2, and 3, respectively. The calculated values for GG in *Columns 9, 10, and 11* are obtained from those found, by correcting for the buffer effect of the amino acids. From these, the values of k for the other peptides have been calculated according to the relative values in *Column 8*. The calculated values are plotted (dashed curves) in Figs. 1, 2, and 3.

* The asterisk indicates that k_2 is greater than k_1 (*i.e.* anhydride formation predominates over splitting).

These are given in Column 4. In Columns 5 and 6 calculated values of $A' = Z(a-B)$ are given in roman type; and observed values of A , in bold faced type. The agreement is very good, except for GG, which was discussed above.

In Column 7 of Table III are the relative values of $k = k_1 + k_2$ as calculated from the dissociation constants (assuming $R_{GG} = 0.026$). They agree qualitatively, but not quantitatively, with those observed.

If, however, we divide the values for SG and GS by 2, and that for SS by 3, we obtain the relative values in Column 8, which agree well with the experimental data. This correction is for some unknown factor which may be "steric hindrance."

With this empirical correction the values of $k = k_1 + k_2$ are calculated for SG, GS, and SS, assuming in each experiment that the curve for GG is correct. The agreement found is as close as could be expected. (See the dashed lines, Figs. 1, 2, and 3.) (See Column 7, Table II, for the observed values of GG and Column 6 for the other peptides.) GS forms an exception.⁴

Thus it will be seen that the splitting and anhydride formation of these peptides with erepsin follow the same laws as with acid hydrolysis. The different action obtained in the different peptides does not indicate any "specificity," but is to be expected on the basis of physical-chemical laws. If the splitting and anhydride formation are produced by two different enzymes, occurring together, it appears that both act on each peptide to an extent predicted above.

In GG, the end-point becomes less sharp (due to amino acid) when $V - V_0$ is about 0.40 to 0.50. With SG and GS this occurs when $V - V_0$ is less than 0.20 (even zero or negative). This is because $V - V_0$ is the resultant ($x - y$) of two processes, hydrolysis (x) and anhydride formation (y). In GG, the former predominates, while in the other cases, the reactions are more nearly balanced. x and y are both large, but the difference ($x - y$) is small. This is direct evidence of simultaneous hydrolysis and anhydride formation.

⁴ The anomalous behavior of GS in both acid hydrolysis and enzyme hydrolysis may be due to the fact that its anhydride is identical with the anhydride of SG and should split more rapidly into the latter. Thus the original material may be contaminated with GS, or there may be some conversion of GS into SG during the reaction.

In Experiment IV the amounts of enzyme and of base were varied in hydrolysis of GG. The rate of splitting was qualitatively, but not quantitatively, proportional to the amount of enzyme solution present. It corresponded approximately to the $\frac{2}{3}$ power of the concentration of enzyme.

While the actual rate of hydrolysis (k values, Table II) of GG with 0.05 equivalent of NaOH was greater than with 0.60 equivalent, the rate (k'), on the basis of the quantity of neutral molecule present, is less. This is to be expected if the enzyme has a dissociation constant whose pK is about 7, and is active on the alkaline side.

EXPERIMENTAL.

The peptides were the same as those used in the first paper and were prepared by Dr. M. H. Pfaltz.

The erepsin was prepared from a stock solution of intestinal juice. NaOH was added to pH 8. The chloroform and toluene which were used as preservatives were removed by extraction with ether. The material was filtered in the cold through a folded filter and the ether was removed in a vacuum desiccator at 0°C. Three solutions were made up. The first was used in Experiment I; the second, in Experiment II; and the third was used in all the other experiments.

0.01 mol of peptide (allowing for moisture) was weighed out, the designated equivalents of normal NaOH were added, 5 cc. of enzyme solution (except in Experiment IV) were added, and the material was transferred to a 10 cc. volumetric flask and made up to 10 cc. at room temperature. A zero reading was taken, and the solutions were placed in a thermostat at 40°C. At the intervals designated, 1.00 cc. samples were taken (correcting the pipette for temperature expansion of the liquid) and titrated against normal NaOH, using tropeolin O as indicator. The alkali was run from a burette with a fine tip, graduated to 0.02 cc. and readable to 0.01 cc.

The results are given in Tables II, IV, and V and Figs. 1 to 3. Time is measured in hours, volume in cubic centimeters.

There is some error involved in estimating the time at which the reaction begins, since some hydrolysis occurs during the time required to dissolve the peptide.

TABLE V.

Experiment IV. Hydrolysis of GG with Different Amounts of NaOH and of Enzyme.

Concentration of NaOH.....	0.3		0.3		0.3		0.6		0.05	
Enzyme solution, cc.....	0.2		1.0		3.0		3.0		3.0	
A (found).....	0.50		0.60		0.84		1.00		a - B	
t	V	100 k	V	100 k	V	100 k	V	100 k	V	100 k
hrs.	cc.		cc.		cc.		cc.		cc.	
0	0.73		0.73		0.74		0.45		1.00	
1	0.74	(1)	0.77	(7.4)	0.79	6.2	0.47	2.1	1.05	(5.3)
2	0.75	1.8	0.77	(3.2)	0.84	6.5	0.50	2.6	1.08	4.1
4	0.77	2.1	0.80	(3.2)	0.93	6.4	0.54	2.3	1.18	(5.3)
5	0.79	2.6	0.86	5.0	0.98	6.7	0.56	2.3	1.29	(4.8)
6	0.81	2.9	0.89	5.3	1.04	6.8	0.58	2.3	1.23	4.6
22.5	0.88	1.6	1.14	5.1	1.43	(7.7)	0.78	1.8	1.60	4.4
29	0.91	1.6	1.16	(4.4)	1.44	6.2				
47	0.97	1.4	1.25	5.5	1.52	6.4				
Average k (equation (8))	2.0		5.1		6.5		2.3		4.4	
k' (equation (9))..	1.4		4.3		7.9		6.3		4.4	

TABLE VI.

Experiment V. Hydrolysis of GG Anhydride. Saturated Solutions (0.20 Molar) with Excess of Solid.

Time, hrs.....	0	1.5	30	7	24	30.5	48
5 cc. of enzyme per 10 cc. 0.015 equivalent of NaOH	0.07	0.11	0.12	0.15	0.20	0.20	0.20
Blank (10 cc. H ₂ O)	0.06	0.06	0.06	0.06	0.08	0.08	
Time, hrs.....	0	1.5	5.5	22	29	46.5	
10 cc. molar HCl.....	1.00	1.03	1.12	1.39	1.45	1.60	

$$k = \frac{\Delta x}{a \Delta t} \text{ for HCl} = 0.10.$$

$$k = \frac{\Delta x}{a \Delta t} \text{ for enzyme} = <0.04 \text{ (probably very small).}$$

(a = 0.20 = solubility of GG anhydride at 40°C.)

Experiment IV, in which the quantities of base and of enzyme were varied, was performed during cloudy weather. The color of the light rendered it impossible to titrate accurately with the indicator used.

Experiment V, on GG anhydride, showed definite splitting with acid, but only slight splitting (if any) with erepsin. The change in titration may have been due entirely to the peptide GG (not in anhydride form) which was present in slight amount, but which was more soluble than the anhydride. Splitting of the anhydride should continue at the same rate as long as solid material is present. Analysis of another solution of anhydride (with only a slight excess of solid) showed the presence of 0.22 mol per liter of anhydride (Kjeldahl). Of this 0.02 mol was not in ring form (by titration). Hence the solubility of the anhydride is 0.20 mol per liter at 40°C.

A 2 molar solution of sarcosine failed to show any synthesis with erepsin (5 cc.) even after 5 days.

SUMMARY.

Erepsin behaves similarly to acid in its action on peptides. Two principal reactions occur simultaneously: (1) hydrolysis, and (2) anhydride formation. If k_1 and k_2 are the velocity constants of these reactions, a formula may be derived (equation (6)) which involves the two constants.

The peptides studied give curves of this type (with one exception). The opening up of anhydride is not promoted as readily by erepsin as by acid.

The strength of any peptide bond, or anhydride bond (as measured by $\frac{1}{k_1}$, and k_2 , respectively) appears to be a function of the acid dissociation constant K_a and of the basic dissociation constant K_b of the two groups involved in the linkage.

Assuming that this "strength" is proportional to $(K_a K_b)^2$, we can calculate values of A' (equation (7)) which correspond well with experimental data. If, in addition, we introduce an empirical correction, values for $k = k_1 + k_2$ may be calculated which agree well with those observed (except in the case of glycyl-sarcosine which behaves anomalously also in acid hydrolysis).

RESEARCHES ON AMINES.

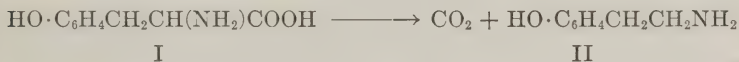
X. THE FORMATION OF TYRAMINE BY DECARBOXYLATION OF TYROSINE PRODUCED FROM SILK.*

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(Received for publication, November 10, 1924.)

The first chemical method for the production of tyramine II was that of Schmitt and Nasse,¹ who obtained it by heating small quantities of tyrosine I to a temperature of 270°. The yield obtained by this method was very poor. Ehrlich and Pistschimuka,² in a later study of the thermal decomposition of tyrosine, attempted to improve the yield of tryamine II by heating the amino acid slowly at 270° under a



pressure of 12 to 25 mm. The tyramine was formed as a sublimate and recovered in the form of its hydrochloride. The application of their method is limited to decomposition of small quantities of tyrosine (1 gm.) and the yield of tyramine is about 50 per cent of the theoretical.

This same decomposition of tyrosine can also be accomplished by biochemical methods. It is produced from tyrosine in the putrefaction of proteins containing this amino acid, but the amount formed in such cases is usually small and the methods of separating and isolating the base in a pure condition are lengthy and cum-

* Constructed from part of a dissertation presented by P. G. Daschavsky to the Faculty of the Graduate School of Yale University, June, 1920, in candidacy for the degree of Doctor of Philosophy.

† Holder of the du Pont Fellowship in Chemistry, 1918-19, and the Loomis Fellowship in Chemistry, 1919-20. Deceased, July 21, 1924.

¹ Schmitt, R., and Nasse, O., *Ann. Chem.*, 1865, cxxxiii, 211.

² Ehrlich, F., and Pistschimuka, P., *Ber. chem. Ges.*, 1912, xlv, 1006.

bersome. Sasaki³ showed that tyrosine can be decarboxylated by the action of *Bacillus coli communis*. The essential features of his procedure are the use of tyrosine and a suitable nutrient medium prepared under sterile conditions to which are added agar-agar colonies of the bacterial organism, freshly isolated from feces and cultivated for 24 hours on agar-agar, and incubated at 37° for 40 days. The tyramine is extracted by means of alcohol and ether and finally purified in the form of its hydrochloride. The yield reported by application of this method with 10 gm. of tyrosine is 78.7 per cent of the theoretical.

Later, Kawai⁴ patented a biochemical process for decarboxylation of tyrosine. In his process he used the bacterium *Bacillus proteus vulgaris* to decompose the amino acid and incubated it for a long time at 37° to obtain complete transformation of the tyrosine to tyramine. As the original Japanese patent literature was not available to us and the abstract was very abbreviated we were unable to obtain the exact details of his method of operating. Apparently his procedure has no advantage over that recommended by Sasaki; also it is impossible even to approximate the cost of production by either method.⁵

The method of decarboxylation that is of immediate interest to us in our work is that devised by Graziani.⁶ This investigator, in an attempt to synthesize cyclo-tyrosyl-tyrosine by heating tyrosine with diphenylmethane, made the very interesting observation that the amino acid was decarboxylated under these conditions and tyramine was produced. Graziani states that his best yield of tyramine was 97 per cent of the theoretical and was obtained by heating a mixture of 1 gm. of tyrosine and 15 cc. of diphenylmethane at 245° for 2½ hours. The tyramine was easily separated after completion of the reaction, and crystallized on cooling, being finally purified by distillation under diminished pressure.

³ Sasaki, T., *Biochem. Z.*, 1914, lix, 429; *J. Biol. Chem.*, 1917, xxxii, 527. Sasaki, T., and Otsuka, I., *J. Biol. Chem.*, 1917, xxxii, 533.

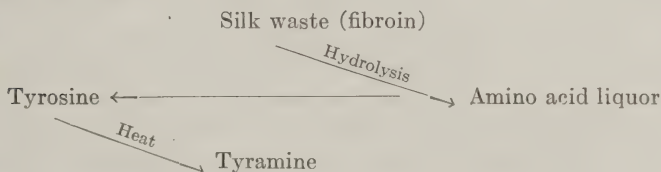
⁴ Kawai, K., Japanese Patent, No. 30,334, November 14, 1916; *Chem. Abstr.*, 1917, xi, 2027.

⁵ See also Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 271; Bettinger, *Bull. assn. chim. suc. dist.*, 1921, xxxviii, 463; *Chem. Abstr.*, 1922, xvi, 1104.

⁶ Graziani, F., *Atti. accad. Lincei*, 1915, xxiv, series 5, pt. 1, 822, 936.

A repetition of Graziani's work by the writers has led to the confirmation of his results. However, if 10 gm. units of tyrosine are used instead of 1 gm., as utilized by this investigator, the decarboxylation period is much longer, 8 to 10 hours being required to complete the reaction. For large scale production this method lacks the proper details and, furthermore, its practical application is dependent on the availability of tyrosine in quantity. His paper, however, furnished us the clue for a new method of operating which has made tyramine a reagent which can now be obtained easily in a pure condition for experimental purposes.

The method which we have developed for the preparation of this interesting base is represented by the scheme given below and is based on the utilization of waste silk (crude fibroin) as a source of the amino acid, tyrosine. Silk gum or sericin, which is also a waste product in the silk industry, is not a suitable protein for tyramine manufacture on account of its low content of tyrosine.



For obtaining the maximum yield of tyrosine from fibroin, Fischer and Skita⁷ recommend the procedure of Weyl,⁸ using 25 per cent sulfuric acid as the hydrolyzing agent. This method we have adopted and it possesses one great advantage over that by hydrochloric acid, in that the acid can be subsequently easily and completely removed by precipitation as barium sulfate. In considering this well known laboratory procedure, it was found necessary, in order to make the method one of practical utility in our work, to substitute another alkali for barium hydroxide, and thereby eliminate the use of carbon dioxide for precipitating barium as carbonate, and finally to displace oil baths and direct heat in operating the digestion or hydrolysis of the protein. These factors of technical improvement have been accomplished as follows: (1) by passing steam through a lead coil immersed in the

⁷ Fischer, E., and Skita, A., *Z. physiol. Chem.*, 1901, xxxiii, 177.

⁸ Weyl, T., *Ber. chem. Ges.*, 1888, xxi, 1529.

acid-protein solution as the method of heating during hydrolysis; (2) by using white lime as the reagent to neutralize sulfuric acid instead of barium hydroxide; and (3) by eliminating the use of carbon dioxide, merely by the reprecipitation and recrystallization of the crude tyrosine.

Decarboxylation of Tyrosine.—While the degradation of α -amino acids to their corresponding amines by bacterial or fungicidal action is well understood and suitable organisms for bringing about this change have been isolated and described, the literature on chemical methods for accomplishing this transformation is very scanty. Cahours⁹ distilled the simpler α -amino acids with lime and baryta and obtained the corresponding amines, but no quantitative data of value are recorded in his publication. Both Schwanert¹⁰ and Limpricht¹¹ merely heated the α -amino acids to obtain the corresponding amines, but here again no data are given regarding yields.

As a general rule dry distillation methods are not productive of good yields, especially when one works with easily carbonized materials. In the specific case of tyrosine, heating the acid alone produces very little tyramine. In heating a crystalline substance of this character, the heat is unevenly distributed so that at one spot, carbonization may take place, while at another, the temperature may be too low to cause decarboxylation. This very problem arose when different investigators tried to make the anhydrides of certain amino acids by heating the solid material. Maillard¹² employed glycerol to moderate the reaction and to increase the yield of degradation products. This procedure proved successful and Maillard assigned this specific effect to the glycerol. Balbiano¹³ concludes, on the other hand, that glycerol exerts no specific action in this change and that anhydride formation is due only to the high temperature used. The glycerol only acts as a diluent and conducts the heat, and, furthermore, may be replaced by other material such as hydrocarbons. Bal-

⁹ Cahours, A., *Ann. Chem.*, 1859, cix, 29.

¹⁰ Schwanert, H., *Ann. Chem.*, 1857, cii, 225.

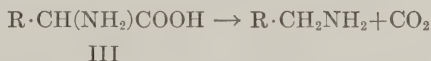
¹¹ Limpricht, H., *Ann. Chem.*, 1857, ci, 297.

¹² Maillard, L. C., *Genese des matieres proteiques et des matieres humiques*, Paris, 1913; *Ann. chim.*, 1914, i, 519; *Chem. Abstr.*, 1914, viii, 1594, 3423.

¹³ Balbiano, L., *Atti accad. Lincei*, 1914, xxiii, series 5, pt. 1, 893.

biano showed that naphthalene and cymene can be utilized successfully in bringing about anhydride formation.

Graziani⁶ confirmed Balbiano's work, extending the observations to diphenylmethane and acenaphthene. In the cases of alanine, leucine, and tyrosine he obtained a smooth conversion to the corresponding amines by heating in the presence of diphenylmethane. Graziani concludes that for each amino acid there is a lower temperature at which anhydrification will be at a maximum, and a correspondingly higher temperature at which, instead, the formation of amines will be at a maximum, but between these two temperatures both reactions are simultaneously possible. It is evident, therefore, that when a given solvent facilitates one type of reaction this will predominate even at a temperature beyond its limits. Thus, glycerol has a great avidity for water and consequently, as a solvent, favors anhydride formation. In general, it may be said, therefore, that the α -amino acid grouping III will evolve carbon dioxide on heating,



substituent groupings (R), of course, influencing the reaction. The yield of resulting amine, however, is a variable factor and is dependent on heat distribution, side reactions, and stability of the amino acid and amine.

After a great many experiments to determine the best solvent for promoting or catalyzing the dissociation of tyrosine into carbon dioxide and tyramine, we have finally adopted a mixture of equal parts of diphenylmethane and diphenylamine. Most excellent results have been obtained by its use and the mixture possesses the following advantages. (1) It remains fluid when cooled to 0°; (2) it has a high boiling point, 260–300°, at atmospheric pressure; (3) the tyramine formed in the reaction is soluble in the hot, but insoluble in the cold solvent, and consequently the latter may be separated from the tyramine by filtration and washing with benzene; (4) the mixture of hydrocarbon and amine is not an expensive reagent to use and the recovery is large; and (5) this special solvent regulates the decarboxylation reaction to give an almost quantitative yield of tyramine (95 to 97 per

cent) in a very short period of heating and with minimum carbonization.

To summarize, we have here the two essential features of a practical method for the production of tyramine from tyrosine; namely, a commercial waste product suitable for the production of tyrosine, and an easy and practical method of converting the latter into tyramine. The success of our method of operating is evidenced by the result obtained in a single experiment when we obtained from 2,000 gm. of silk noils (crude fibroin) 94 gm. of pure, colorless tyramine hydrochloride.

EXPERIMENTAL PART.

Hydrolysis of Fibroin.—For the preparation of tyrosine from fibroin we have, in agreement with Fischer, found that 20 to 25 per cent sulfuric acid is a very suitable reagent for hydrolyzing the protein. The hydrolysis was carried out in a lead-lined vessel of about 40 liters capacity, and the heating done by means of an immersed lead coil, through which steam circulated. This vessel was covered by a lead top containing openings for the ends of the immersed coil and a small aperture for sampling purposes.¹⁴ To explain the details of our method of operating we will give a description of a single experiment.

Composition of Mixture.

Silk noils (crude fibroin).....	2,000 gm.
Concentrated H ₂ SO ₄	4,000 cc.
Water.....	20,000 "

Procedure.

The 20 per cent H₂SO₄ was poured into the kettle with the lead coil in position, and the silk noils were added in portions of 200 gm. Care must be taken to insure thorough wetting of the noils by the acid. The lead cover was then placed in position and steam allowed to circulate through the coil for about 60 hours. Small samples of the material were withdrawn from time to time and subjected to the biuret test, which was used as a criterion for judging the completeness of hydrolysis of the protein. When the

¹⁴ All the silk noils (crude fibroin) used in this investigation were furnished gratuitously by Cheney Bros., Silk Manufacturers of South Manchester, Conn.

mixture no longer gave the biuret coloration, the heating was stopped and 20 liters of water were added. The diluted acid liquor was then neutralized with finely powdered lime, using a motor-driven stirrer to agitate the contents of the kettle, the neutral mass filtered by suction on large porcelain suction filters, and the precipitate of CaSO_4 washed with 10 liters of hot water. After removing the CaSO_4 from the filter, it was then digested with 90 liters of boiling water in six portions, using the motor stirrer for agitation, and at the same time blowing steam through the mass, then allowed to settle, and filtered. After careful washing of the CaSO_4 with hot water the combined filtrates were added to the original mother liquor and the solution was evaporated in an enameled pan to a volume of about 15 liters. At this point, the liquid was again tested for neutrality and allowed to stand overnight. Tyrosine which separated was filtered and washed with cold water. The filtrate at this point contains the residual amino acids of the protein and is saved.¹⁵

The tyrosine which separates on standing contains some CaSO_4 as impurity, and to purify the acid it was digested on the steam bath successively with two portions of an alkaline solution containing 500 gm. of NaOH in 2 liters of water. This treatment served to dissolve the tyrosine away from the lime impurities. The alkaline liquor was then filtered by suction, washed with water, the filtrate diluted with a liter of water, and neutralized exactly with hydrochloric acid, using Congo red as an outside indicator. At the neutral point tyrosine precipitated and was filtered by suction and washed thoroughly with cold water. To obtain the amino acid in a colorless and pure condition, the crude material was dissolved in 20 liters of boiling water containing some "norit" as decolorizing agent, filtered hot, and allowed to crystallize. The pure tyrosine was then filtered, washed with cold water, and dried at 100° . From 2,000 gm. of fibroin we obtained 140 gm. of the pure amino acid, equivalent to a yield of 7 per cent. Many hydrolysis experiments with varying amounts of noils confirmed this result.

The silk noils (fibroin) used in our research were derived from Chinese Canton silk, and the yield of tyrosine obtained (7 per cent) compares very favorably with the quantitative result (9.8 per cent) as recorded by Plimmer¹⁶, and expressed in Table I.

Conversion of Tyrosine into Tyramine.—The basis for the following experimental development is the interesting result obtained by Graziani in which tyramine is produced from tyrosine by heating with diphenylmethane. Of the various organic solvents tested for their power of decarboxylating this amino acid, an equal mix-

¹⁵ Uses for this valuable amino acid liquor are being developed (T. B. Johnson).

¹⁶ Plimmer, R. H. A., *Chemical constitution of the proteins*, Monographs on biochemistry, London, New York, Bombay, and Calcutta, 2nd edition, 1912-13.

ture of diphenylamine and diphenylmethane was found to give the most satisfactory results. A description of a single experiment will illustrate the technique of our method of operating.

20 gm. of tyrosine were mixed with 120 cc. of pure distilled diphenylmethane and 120 gm. of diphenylamine in a liter round bottom Pyrex flask, fitted with an air condenser, and the mixture was carefully heated with a Bunsen flame, care being taken to prevent local superheating or charring. At 260° carbon dioxide began to be liberated and in a few minutes the evolution of this gas was copious; and at the end of 40 minutes heating at a temperature of 260–265°, the reaction was apparently completed, the hydrocarbon-amine solution presenting a clear yellow appearance without any indication of carbonization. Although the melting point of tyrosine is about 295° our decarboxylation had been effected in this medium at 260°. The clear yellow solution was allowed to cool to 60° when the mass became

TABLE I.

Yield of Tyrosine by Hydrolysis of Fibroin from Different Sources.

Silk fibroin.	Tyrosine.
	<i>per cent</i>
Italian.....	10.5
Chinese, New Chwang.....	9.8
" Canton.....	9.8
" Shantung.....	9.7
" Niet-ngo-Tsam.....	7.8
" Tai-Tsao-Tsam.....	7.8
" Cheefoo.....	8.5
" Tailung.....	3.6
Indian, Bengal.....	10.0
" 	9.2

quite cloudy. At this point 100 cc. of benzene were added to prevent tyramine from adhering to the walls of the flask, and the contents agitated to cause thorough mixing of the benzene. 1 hour cooling in an ice bath caused the tyramine to deposit as a fine yellow powder which was filtered off by suction and washed several times with warm benzene to remove the solvent mixture. What tyramine adhered to the walls of the reaction flask was recovered by conversion to the hydrochloride and extracted with water.

From 140 gm. of tyrosine thus treated in seven runs of 20 gm. each, there were obtained 86 gm. of crude tyramine and 18.5 gm. of crude tyramine hydrochloride, equivalent to 14.6 gm. of the base, giving a total of 100.6 gm. of crude tyramine. A quantitative yield of tyramine from tyrosine is 105.98 gm. Therefore, our

yield of tyramine is equivalent to 95 per cent of the theoretical, a result which establishes this method of preparation as the most productive and practical of any hitherto described.

The crude tyramine was digested with 300 cc. of concentrated hydrochloric acid and 600 cc. of water, the solution filtered and then concentrated by evaporation *in vacuo* on the steam bath to incipient crystallization, and then quickly poured into a beaker and allowed to crystallize. A second crop of the hydrochloride was obtained by further concentration of the acid liquor. The hydrochloride was finally purified by recrystallization from hot absolute alcohol, strongly acidified with hydrochloric acid gas, when it separated, on cooling, as colorless glistening needles. Three crops of the hydrochloride were collected as follows:

1st.....	66 gm.	melting at 269–270°
2nd.....	20 “	“ “ 268.5–269.5°
3rd.....	8 “	“ “ 267.5–268°
Total.....	94 “	tyramine hydrochloride.

A portion of the first fraction was again crystallized from absolute alcohol containing hydrochloric acid and, when dried *in vacuo* over concentrated sulfuric acid for 48 hours, had the following properties.

It melted at 269.5–270° to a clear liquid. Koessler and Hanke¹⁷ give 280° as the melting point of their tyramine hydrochloride containing 1.86 per cent of sodium chloride as impurity. Barger¹⁸ reports a melting point of 268°. Analysis: 0.3152 gm. of tyramine hydrochloride ignited in a weighed platinum crucible at a red heat left no weighable residue. 0.4860 gm. of tyramine hydrochloride when treated in aqueous solution with AgNO₃ gave 0.4001 gm. of AgCl instead of the theoretical amount or 0.4007 gm. Nitrogen determination (Kjeldahl method):

C ₈ H ₁₁ ON·HCl.	Calculated.	N 8.07.
	Found.	“ 8.00, 8.01.

Recovery of Solvents.—The benzene used for washing the tyramine can easily be separated from the solvent mixture by dis-

¹⁷ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 585.

¹⁸ Barger, G., The simpler natural bases, Monographs on biochemistry, London, New York, Bombay, and Calcutta, 1914, 10, 18; *J. Chem. Soc.*, 1909, xcv, 1123; English Patent, No. 1,560, 1909. Barger, G., and Walpole, G. S., *J. Chem. Soc.*, 1909, xcv, 1720; English Patents, Nos. 1,561 and 17,171, 1909.

tillation under diminished pressure. The resulting liquid consisting of diphenylamine and diphenylmethane, when fractionally distilled *in vacuo*, is easily purified for further work, giving a satisfactory and clean-cut separation.

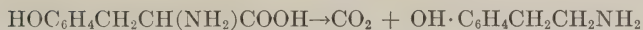
Rejected Solvents.—In testing the various solvents for decarboxylating tyrosine, a specially constructed apparatus was employed which enabled us to determine quantitatively the amount of CO₂ generated from a known quantity of tyrosine. By weighing the evolved CO₂ a relative measure of the catalytic influence of solvents tested could be determined without isolating the tyramine formed. The solvents tested were paraffin, white vaseline, aniline, quinoline, α -naphthylamine, anthracene, diphenylmethane, diphenylamine, and diethylaniline.

Paraffin was found to promote decomposition of tyramine, but it was very difficult to separate the base from this solvent. Vaseline was also rejected for the same reasons. Aniline, which allowed of heating only to 183°, did not cause decarboxylation of the amino acid. Quinoline when heated to its boiling point dissolved tyrosine quickly. However, the tyramine, if formed, does not crystallize on cooling and separation of quinoline and tyramine is not accomplished easily.

Diethylaniline did not convert tyrosine to tyramine in any satisfactory manner under the maximum temperature conditions; namely, 216°. A temperature of about 250–260° was found to be necessary to decarboxylate the tyrosine, and an inspection of the boiling points of aniline, quinoline, and diethylaniline shows them to lie below the minimum temperature required for decomposing the amino acid. The use of α -naphthylamine was prohibited because of the tar formed and the difficulty in isolating the tyramine formed. In fact, no pure tyramine could be isolated. Anthracene was found to be valueless as a solvent because of its high melting point (217°) and great tendency to sublime.

SUMMARY.

1. A practical method has been developed for the production of the important, active amine, tyramine, from silk fibroin.
2. The method developed has several advantages over those previously described in the literature, and is based on the well known behavior of tyrosine when heated above 260°.



3. Utilization is made of a trade waste product (silk noils), and the complete synthesis is accomplished in two major operations; namely, (*a*) hydrolysis of silk fibroin to produce the amino acid, tyrosine, and (*b*) decarboxylation of the tyrosine to tyramine by heating at 260° in a catalytic medium composed of equal parts of diphenylmethane and diphenylamine.

4. This investigation has revealed the importance of a more thorough study of catalytic reagents which facilitate the formation of amines by heating amino acids.

FAT ABSORPTION THROUGH CHANNELS OTHER THAN THE LEFT THORACIC DUCT.*

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(Received for publication, November 25, 1924.)

The absorption of fat from the intestinal tract is unique in as far as the major portion, if not all, of the products involved enters the general circulation by way of the thoracic duct. In this respect fats differ markedly from the digestion products of the proteins and carbohydrates which are, for the most part, absorbed into the capillaries of the portal circulation. Investigations carried out by Munk and Rosenstein (1), showing that no more than 60 per cent of the ingested fat could be recovered from the lymph, have suggested that a part of the remainder at least may find its way into the body through the portal circulation. This conception is supported by the researches of Munk and Friedlander (2) who found a sixfold increase in the total fatty acid content of the blood after fat had been ingested by dogs in which the thoracic duct had been ligated. Additional favorable evidence was presented by Hamburger (3) who reported a disappearance of fat introduced into isolated loops of the small intestine from which all visible lymphatics had been tied off. On the other hand, Bloor (4) was unable to confirm the findings of Munk and Friedlander, and Fish (5), making use of the dark-field microscope, failed to observe more fat particles in the portal blood than were present in samples of blood taken simultaneously from the jugular vein. Bloor's apparent contradiction of the findings of Munk and Friedlander might be ascribed to a rapid transport of fat from the blood to the tissues.

* The data are taken from the dissertation submitted by Henry C. Eckstein in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate Faculty of Yale University, June, 1923.

With regard to the report of Fish, it is likely, as he himself has pointed out, that some of the blood fat escapes detection with the microscope.

The writer is of the opinion that the one experiment reported by Bloor needs confirmation, inasmuch as no evidence was presented to prove that fat had actually been absorbed. In view of this, a series of experiments was conducted in which samples of lymph as well as blood were taken from dogs before and after the introduction of neutral fat into the duodenum. The subsequent analyses of the blood and lymph thus afforded a means of determining with certainty whether fat absorption had actually taken place and whether some of the absorbed fat had entered the body through paths other than the diverted lymph.

EXPERIMENTAL PROCEDURE.

Dogs that had been fasted for at least 48 hours were anesthetized with an A.C.E. mixture.¹ Cannulas were then introduced into the thoracic duct and femoral artery. After samples sufficient for preliminary analysis had been obtained, olive oil emulsified with desiccated ox-gall and gum acacia was injected into the duodenum and the collection of blood and lymph samples continued for as long a time as the animal survived. In order to compensate for the fluid removed, a volume of normal saline solution approximating the volume of lymph collected during each 2 hour period was injected intravenously or subcutaneously as soon as possible after the taking of blood samples. Inasmuch as the volume of saline solution injected rarely exceeded 50 cc., and, furthermore, since 2 hours were allowed to elapse before the subsequent samples of blood were taken, the effect of the saline injection on the blood volume was considered negligible. This effect was consequently not taken into consideration in the calculation of the results obtained. Control experiments were conducted in which, with the exception of the introduction of neutral fat, the details noted above were exactly duplicated.

The modified method of Bloor (6) which gives results that agree well with those obtained with gravimetric procedures was used in the determination of the total fatty acid content of the blood and lymph.

¹ Acetone-chloroform-ether mixture.

DISCUSSION.

It is evident from a study of results summarized in Table I that even when all of the thoracic lymph is diverted, as described, from the blood stream, an appreciable though small augmentation of the total fatty acid content of the blood follows the absorption of neutral fat from the duodenum. It is likewise apparent, as shown in Tables II and III, that marked increases in the lymph fat occurred when fat was being absorbed. Experiment 6 (Table I) is of interest in that it shows clearly that fat had failed to be absorbed. Under this circumstance, no changes in the blood fat were to be expected, provided, of course, that the deep anesthesia employed would not invoke a lipemia. That this is the case is made clear from Tables IV and V in which the control experiments are summarized. The results obtained in Experiment 9 (Tables I and II) are contrary to what might be expected in the light of the other experiments and taken by themselves might be submitted as evidence favoring the view that none of the absorbed fat enters the body by paths other than the thoracic lymph. The writer feels, however, that an alternate view which postulates a transport of fat from the blood to the tissues at a rate equivalent to the rate of its absorption from the intestine may better explain this isolated experiment, especially since the remaining experiments adequately favor the supposition that there are channels other than the lymph stream which may serve as a vehicle for the transportation of fat into the body. The writer, however, hesitates to conclude that fat is directly absorbed by the portal capillaries inasmuch as all of the lymphatics may not necessarily be ruled out by merely diverting the thoracic trunk lymph from the body.

TABLE I.

Changes in the Total Fatty Acid Contents of the Whole Blood after Feeding Fat to Dogs Bearing a Lymph Fistula.

Time.	Experiment No.					
	5	6	9	10	11	12
	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>
Before.	0.60	0.52	0.47	0.40	0.50	0.50
After.						
1 hr.	0.57	0.54	0.47	0.59	0.64	0.51
3 hrs.	0.66	0.58	0.50	0.61	0.67	0.57
5 "	0.63	0.52	0.49	0.64	0.68	0.68
7 "	0.88		0.52			0.71
9 "	0.81		0.51			0.58
11 "	0.80		0.50			0.56
13 "	0.80					
15 "	0.79					

TABLE II.

Changes in the Total Fatty Acid Content of the Thoracic Lymph of Dogs after Introduction of Olive Oil into the Small Intestine.

Time.	Experiment No.					
	5	6	9	10	11	12
	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>
Before.	0.28	0.32	0.23	0.30	0.35	0.38
After.						
0 to 2 hrs.	0.27	0.28	0.54	0.62	0.78	0.33
2 " 4 "	0.48	0.32	1.05	0.80	1.48	0.55
4 " 6 "	1.55	0.37	1.94	1.51	1.40	1.02
6 " 8 "	1.67		1.62			1.80
8 " 10 "	1.70		0.84			2.20
10 " 12 "	1.70		0.72			1.10
12 " 14 "	2.40					
14 " 15 "	1.72					

TABLE IV.

Effect of the Anesthesia on the Total Fatty Acid Content of the Thoracic Lymph of Fasting Dogs.

Time after starting anesthesia.	Chloroform. Experiment 1.		A. C. E.* Experiment 2.		A. C. E.* Experiment 3.	
	Fatty acids.	Lymph volume.	Fatty acids.	Lymph volume.	Fatty acids.	Lymph volume.
hrs.	gm.	cc.	gm.	cc.	gm.	cc.
2 to 3	0.24	15	0.29	38	0.29	63
3 " 5	0.25	32	0.29	41	0.28	28
5 " 7	0.26	29	0.26	26	0.30	15
7 " 9	0.24	33				
9 " 11	0.27	21				
11 " 13	0.26	18				

* Alcohol-chloroform-ether mixture.

TABLE V.

Effect of the Anesthesia on the Total Fatty Acid Content of the Blood of Fasting Dogs Bearing a Lymph Fistula.

Time after starting anesthesia.	Fatty acids.		
	Chloroform. Experiment 1.	A. C. E.* Experiment 2.	A. C. E.* Experiment 3.
hrs.	gm.	gm.	gm.
2	0.61	0.36	
3	0.60	0.42	0.43
5		0.40	
6	0.59	0.42	0.44
7		0.38	0.42
8	0.65		
10	0.66		
12	0.60		

* Alcohol-chloroform-ether mixture.

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SYNTHESIS OF LECITHIN IN THE ANIMAL ORGANISM.*

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The generally accepted view that the hydrolysis products of the fats in their transport across the intestinal wall recombine with the formation of triglycerides has led to the question as to the mechanism whereby such water-insoluble products as the neutral fats are transported in the circulating fluid to the tissues. A detailed study of this question has recently led Bloor (1) to postulate the view that the formation of lecithin, a substance quite miscible with water, might facilitate the transport of fat within the body.

The similarity between the conventional structural formula of a lecithin and a triglyceride favors the view that the former may be synthesized from the latter. Lecithin synthesis within the organism is not unknown. McCollum, Halpin, and Drescher (2) proved it to take place in the growth of chickens; while Drosdoff (3) found that the blood as it passes through the liver gains in organic and loses in inorganic phosphorus, indicating that a synthesis of the former had taken place at the expense of the latter. Furthermore, Meigs, Blatherwick, and Carey (4) demonstrated the reversibility of the reaction in their observations that all of the milk fat produced by the dairy cow can be accounted for by the differences in the lipoid phosphorus coming to and leaving the mammary gland. In studying the question further Bloor (1) found in dogs that an increase in the total fatty acid and lecithin content of the whole blood takes place after fat

* The data in this paper are taken from the dissertation submitted by Henry C. Eckstein in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Graduate Faculty of Yale University, June, 1923.

is ingested. Since the phosphatide augmentation is often not marked until a considerable increase in the total fatty acid content of the blood has taken place, and, furthermore, since this augmentation is most noticeable in the corpuscles, Bloor (1) concluded that the erythrocytes possess the power of absorbing fat from the plasma and of transforming it into lecithin. Studies of lipemias produced in other ways add weight to Bloor's hypothesis. In diabetes the increase in the fat content of the plasma and corpuscles is accompanied by a similar augmentation of the lecithin (Bloor, 5). In severe cases of pernicious anemia, though the total fatty acid content of the blood cells is normal, the corpuscular lecithin is below normal. According to Bloor and MacPherson (6) this is probably due to the fact that the lack of so many corpuscles markedly affects the synthesizing powers of the organism. A more recent consideration of this question (7) led Bloor to the conclusion that lecithin synthesis is not entirely limited to the erythrocytes but that it may occur in many other body cells.

The facts now available indicate a sufficiently close relationship between the fatty acids and subsequent lecithin increments of the blood to give an important rôle to the phosphatides in the transport of fat within the body. The data pointing to the synthesis of lecithin by the blood corpuscles are, however, not so convincing as might be desired, inasmuch as the evidence thus far presented is just as favorable to the view that lecithin synthesis takes place during the transport of the hydrolysis products of the fats through the intestinal mucosa and that the erythrocytes merely function as carriers of the synthesized phosphatide.

The investigation reported herein was undertaken to throw light upon the relative significance of the different hypotheses.

EXPERIMENTAL PROCEDURE.

Owing to the peculiar anatomical arrangement whereby most of the fat absorbed can go directly into the lacteals it is possible, from a chemical examination of the thoracic lymph, before and after the absorption of fats of known composition, to state with certainty whether the small intestine takes part in the synthesis of lecithin. With this in mind several groups of experiments

were planned. The first of these consisted in determining the total fatty acid and lecithin contents of the thoracic lymph before and after the absorption of neutral fats from the alimentary tract. Dogs that had been fasted at least 48 hours were anesthetized, and a cannula then introduced into the thoracic duct. After a sample of lymph sufficient for preliminary analysis had been obtained, olive oil emulsified with desiccated ox-gall and gum acacia was injected into the duodenum and the collection of lymph samples continued for as long a time as the animal survived. In order to compensate for the fluid withdrawn from the body a volume of normal saline solution approximating the volume of lymph removed during each collection period was injected intravenously as soon as possible thereafter. In some of the later experiments the saline solution was injected subcutaneously.

Since it seemed possible that overwhelming amounts of fat might stimulate a synthesis of phosphatides which might not take place when only moderate amounts of neutral fat were introduced into the small intestine, the intake of olive oil was increased from the 5 cc. used in five experiments to 20 cc. in two others. Finally two were performed in which 100 cc. of the oil were given by sound 2 hours previous to the operation. In view of the fact that numerous analyses of the preliminary lymph samples of different dogs showed that the total fatty acid and lecithin contents of the lymph collected after a 48 hour fast varied within only narrow limits, it was considered needless to make the collection of the lymph in the fore periods of these latter experiments.

A second group of experiments differed from the first in two respects: (1) varying amounts of pure fatty acids were substituted for the olive oil; (2) these were *fed* to the dogs 2 hours before the operation. The fatty acids used differed from each other in that the one was the liquid and somewhat *toxic* oleic acid, whereas the other, palmitic, was solid at body temperature and *non-toxic*. It was found desirable to incorporate the acids into a diet consisting of gelatin and meat extract, because attempts to feed them without admixture invariably resulted in failure.

A similar procedure was also adopted in the third group of experiments in which egg lecithin was fed. In one experiment

(Experiment 14) the entire yolk of fresh eggs was employed as the source of lecithin; in the subsequent three experiments lecithin isolated from egg yolk was substituted for the fatty acids in the diets described above.

In a series of control experiments the injection of fats or fat derivatives into the duodenum was omitted; otherwise, the details noted above were closely followed.

The analytical methods employed were as follows: Total fatty acids were determined by the method devised by Bloor, Pelkan, and Allen (8). During the early course of the investigation Bloor's nephelometric method (9) for lecithin was used. This method was later replaced by the colorimetric procedure recently outlined by Randles and Knudson (10). The change was, however, not made until it was shown that analyses obtained with one method duplicated those secured with the other. Baumann (11) reports that the method of Bloor as well as that of Randles and Knudson gives results that are approximately 5 per cent lower than results obtained by his modified procedure of the method of Randles and Knudson. The method used in this investigation is, nevertheless, sufficiently accurate to follow the changes taking place in the lymph in an investigation such as this where even a 10 per cent variation in the lecithin of the lymph is negligible.

DISCUSSION.

It will be observed from the results summarized in Tables I and III that the total fat content of the thoracic lymph (always measured as fatty acid) increases rapidly after the introduction of neutral fat into the duodenum. On the other hand, it is quite evident that no appreciable alterations in the lymph lecithin content are brought about as the result of the absorption of fat. This condition, which is clearly demonstrated by the experiments grouped under Table II, would not ensue if a transformation of the digestion products of the fats to lecithin took place in the intestinal mucosa. Hence *it is unlikely that a phosphatide synthesis occurs before the fat enters the blood stream.*

A further study of the tables warrants the conclusion that the total fatty acid and lecithin contents of the lymph of different dogs after a 48 hour fast vary within certain narrow limits. In

this respect the lymph is similar to the blood. The former differs from the latter, however, in that during the preabsorptive

TABLE I.

Changes in the Total Fatty Acid Content of the Thoracic Lymph of Dogs after Introduction of Olive Oil into the Small Intestine.

Time.	Experiment No.							
	5	6	9	10	11	12	13	15
hrs.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Before.	0.28	0.32	0.23	0.30	0.35	0.38		
After.								
0 to 2	0.27	0.28	0.54	0.62	0.78	0.33		
2 " 4	0.48	0.32	1.05	0.80	1.48	0.55	2.37	2.70
4 " 6	1.55	0.37	1.94	1.51	1.40	1.02	2.75	2.05
6 " 8	1.67		1.62			1.80	2.36	1.66
8 " 10	1.70		0.84			2.20	1.77	1.34
10 " 12	1.70		0.72			1.10	1.25	0.86
12 " 14	2.40						1.11	0.44
14 " 15	1.72						0.44	

TABLE II.

Lecithin Changes in the Thoracic Lymph of Dogs after the Introduction of Olive Oil into the Small Intestine.

Time.	Experiment No.							
	5	6	9	10	11	12	13	15
hrs.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Before.	0.16	0.16	0.19	0.21	0.27	0.24		
After.								
0 to 2	0.18	0.17	0.19	0.23	0.23	0.24		
2 " 4	0.18	0.15	0.20	0.21	0.20	0.25	0.21	0.20
4 " 6	0.21	0.16	0.21	0.20	0.20	0.28	0.21	0.20
6 " 8	0.22	0.16	0.19			0.27	0.21	0.20
8 " 10	0.23		0.21			0.27	0.18	0.23
10 " 12	0.20		0.20			0.29	0.23	0.19
12 " 14	0.20						0.23	0.22
14 " 15	0.20						0.20	

period the actual percentage of the lipins is only about half as high in the lymph as in the blood.

Considerably less fat was found to enter the body through the

lymphatics than was to be expected in the light of the studies of Munk and Rosenstein (12) who observed that 15 hours after the ingestion of fat by a patient bearing a lymph fistula over 37 per cent of the fat fed had passed into the lymph. Under the conditions of the experiments of this investigation, *i.e.* deep and

TABLE IV.

Total Fat Recovered in the Thoracic Lymph Calculated as Percentage of Fat Introduced into the Small Intestine.

Experiment No.	Fat introduced.	Duration of experiment.	Amount recovered.
	<i>gm.</i>	<i>hrs.</i>	<i>per cent</i>
5	20	15	11
9	5	12	21
10	5	6	9
11	5	6	17
12	20	12	4
13	100	15	6.5
15	100	14	14

TABLE V.

Changes in the Total Fatty Acid Content of the Thoracic Lymph of Dogs after Feeding Pure Fatty Acids.

Time.	Experiment No.				
	16 Oleic acid.	17 Oleic acid.	19 Palmitic acid.	20 Palmitic acid.	23 Palmitic acid.
<i>hrs.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>
Before.	0.31	0.31	0.31	0.31	0.31
After.					
2 to 4	2.03	2.10	1.39	1.82	2.00
4 " 6	1.05	1.50	0.57	1.50	1.57
6 " 8	0.75	0.82	0.54	1.16	1.61
8 " 10	0.61	0.76	0.38	1.06	1.02
10 " 12	0.24	0.70	0.34	0.92	0.91

prolonged anesthesia, the writer did not expect as good absorption of fat as under more normal conditions. The results tabulated in Table IV show to what extent these differ from what is usually considered normal. With the exception of Experiment 12, as good absorption of fat was obtained as could be expected from experiments in which such factors as deep anesthesia and surgical shock are likely to play an interfering rôle.

The results summarized in Tables V and VI may at first sight be considered as suggestive of a synthesis of lecithin from ingested fatty acid. The writer is of the opinion, however, that the lecithin increases noted in Experiments 16, 17, and 19 may be ascribed to a cause other than the transformation of the ingested products to phosphatides, for the reason that evidences of toxemia were obtained in the experiments in question. In all of these cases the lymph was extremely yellow in color. This observation recalls the researches of Faust and Tallquist (13) who demonstrated that unsaturated fatty acids such as oleic are toxic; hence disturbances were to be expected from the ingestion of

TABLE VI.

Changes in the Lecithin Content of the Thoracic Lymph of Dogs after the Feeding of Pure Fatty Acids.

Time.	Experiment No.				
	16 Oleic acid.	17 Oleic acid.	19 Palmitic acid.	20 Palmitic acid.	23 Palmitic acid.
hrs.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Before.	0.20	0.20	0.20	0.20	0.20
After.					
2 to 4	0.42	0.29	0.26	0.28	0.34
4 " 6	0.50	0.33	0.27	0.25	0.30
6 " 8	0.51	0.37	0.33	0.24	0.26
8 " 10	0.69	0.43	0.41	0.26	0.20
10 " 12	0.17	0.48	0.43	0.27	0.20

large quantities of it. It was at first somewhat surprising to note an abnormal coloration of the lymph in Experiment 19 in which palmitic acid was fed, inasmuch as Faust and Tallquist showed that the saturated fatty acids are non-toxic. The autopsy, however, revealed numerous worms in the small intestine. According to Faust and Tallquist (13) these parasites owe their hemolytic action to their content of a cholesterol ester of oleic acid. When it is considered that no unusual coloration of the lymph was observed in the subsequent palmitic acid experiments in which autopsy showed an absence of intestinal parasites, it is safe to assume that the abnormality noticed in the one experiment was due to the worms. Since a yellow color in body fluids of carnivora is often suggestive of bile, tests for this product

were made in the lymph collected in all of the fatty acid experiments. As was expected, an exact correlation between the yellow color and bile pigments was found. A second parallelism between yellow color and abnormalities in the lymph is to be found in the unusually high lecithin figures obtained in all of the oleic acid experiments and in the one palmitic acid experiment in which the parasites were found in the duodenum. This increased amount of lecithin in the lymph cannot, however, be due entirely to the bile, because of the insufficiency of lecithin in the secretion to account for even the slight augmentation noted in the lymph. Hence it is possible that the oleic acid experiments may be regarded as suggestive of a lecithin synthesis in the small intestine. The writer is, however, rather of the opinion that these lecithin increments may be ascribed to other causes, inasmuch as no verification of the suggestion of a lecithin synthesis was obtained from the properly controlled palmitic acid experiments. There is no reason to suppose that a synthesis of lecithin should proceed with greater ease from oleic acid than from palmitic acid. The fact that Faust (14) found it necessary to feed oleic acids in amounts as high as 10 gm. per day for periods more than a month in length before a hemolysis sufficient to be observed by a decrease in the number of red cells in the blood, suggests that the toxemia noted above may be ascribed to a cause other than the hemolytic power of oleic acid.

The variations in the total fatty acids in the lymph affected by the ingestion of fatty acids parallel those observed when olive oil was fed to dogs. This is clearly demonstrated by the results summarized in Tables V and VII. Differences between the amounts of fatty acids recovered in the oleic acid experiments and those recovered when palmitic acid was fed were rather expected, because the one, oleic acid, is a liquid at body temperature and soluble in the bile of dogs; the other, however, is solid at body temperature and is, according to Moore and Rockwood (15), insoluble in dog bile. The differences between the amounts absorbed are shown in Table VIII.

In view of the fact that the animals were under deep anesthesia and that some fatty acid, at least, had entered the lacteals before the collection of lymph was begun, a confirmation of Munk and Rosenstein's (12) conclusion that 45 per cent of the ingested

TABLE VII.
Description of Thoracic Lymph Samples Collected in Experiments Grouped in Tables V and VI.

Time.		Experiment No. 2											
		16		17		19		20		23			
		Volume.	Appear- ance.	Volume.	Appear- ance.	Volume.	Appear- ance.	Volume.	Appear- ance.	Volume.	Appear- ance.		
hrs.		cc.		cc.		cc.		cc.		cc.			
Before.		89	Creamy.	89	Creamy and yellow.	88	Creamy and yellow.	10	Creamy and white.	30	Creamy and white.		
After.													
2 to 4		55	"	45	"	49	Milky	40	"	35	"		
4 " 6		47	Milky and yellow.	40	"	51	"	35	"	60	"		
6 " 8		32	"	38	"	47	Clear	36	"	51	"		
8 " 10		35	"	35	"	7	"	10	"	40	Milky		
10 " 12		265	Clear							15	"		

fatty acids can enter the body by way of the lymph stream was not to be expected. A comparison of Tables IV and VIII shows that the amounts recovered in the lymph in the olive oil experiments are considerably more than those recovered when fatty acids are fed. This is in strict accordance with the observations made by Munk and Rosenstein (12) on man.

Experience has taught that fatty acids, their simple esters, and the monoglycerides are transformed into triglycerides by the intestinal mucosa. In the light of the observations of Bókay (16) that ample provision is made in the gastrointestinal tract for the hydrolysis of the phosphatides a synthesis of neutral fat might be expected as the result of the feeding of the diglyceride, lecithin. On the other hand, if the intestinal mucosa

TABLE VIII.

Total Fatty Acids Recovered in the Thoracic Lymph Calculated as Percentage of the Fatty Acids Fed.

Experiment No.	Acid fed.	Duration of experiment.	Amount recovered.
		<i>hrs.</i>	<i>per cent</i>
16	Oleic.	12	4.4
17	"	12	5.2
19	Palmitic.	12	3.8
20	"	12	2.2
23	"	12	2.6

possesses the power of synthesizing phosphatides it certainly should exhibit this property when all of the essential radicles required for such a conjugation are furnished it; *i.e.*, by feeding the substance in question.

There is no good reason to believe from the results shown in Tables IX and X that lecithin absorption differs in any manner from the absorption of other fatty acid esters. The marked increase in the total fatty acid content of the lymph that occurs when neutral fats are fed accompanies the absorption of ingested lecithin. A more detailed analysis of the combined lymph samples obtained in Experiment 22 showed that the chyle fat consisted for the most part of triglycerides. Hence it is evident that a synthesis of neutral fat had taken place during the transport of the digestion products of lecithin through the intestinal wall. A more recent study has led Eichholtz (17) to conclude that such

a transformation can take place only to a very slight degree. The evidence upon which this conclusion was based is, however, not convincing, inasmuch as it depends entirely on the fact

TABLE IX.

Changes in the Total Fatty Acid Content of the Thoracic Lymph during the Absorption of Egg Lecithin.

Experiment 14.			Experiment 21.			Experiment 22.			Experiment 24.		
Time.	Fatty acids.	Lymph volume.	Time.	Fatty acids.	Lymph volume.	Time.	Fatty acids.	Lymph volume.	Time.	Fatty acids.	Lymph volume.
hrs.	gm.	cc.	hrs.	gm.	cc.	hrs.	gm.	cc.	hrs.	gm.	cc.
Before.	0.31		Before.	0.31		Before.	0.31		Before.	0.31	
After.			After.			After.			After.		
2 to 4	2.95	63	3 to 4	1.74	27	3 to 3½	1.06	15	3 to 3½	1.69	8
4 " 6	2.75	47	4 " 5	1.02	26	3½ " 4	0.84	16			
6 " 8	1.76	37	5 " 6	1.20	24	4 " 5	0.59	38			
8 " 10	1.68	31	6 " 7	0.49	27	5 " 6	0.67	35			
10 " 12	1.72	15	7 " 8	0.36	20	6 " 6½	0.76	10			
						6½ " 7	1.05	19			

TABLE X.

Changes in the Lecithin Content of the Thoracic Lymph during the Absorption of Egg Lecithin.

Experiment 14.			Experiment 21.			Experiment 22.			Experiment 24.		
Time.	Lecithin.	Lymph volume.	Time.	Lecithin.	Lymph volume.	Time.	Lecithin.	Lymph volume.	Time.	Lecithin.	Lymph volume.
hrs.	gm.	cc.	hrs.	gm.	cc.	hrs.	gm.	cc.	hrs.	gm.	cc.
Before.	0.20		Before.	0.20		Before.	0.20		Before.	0.20	
After.			After.			After.			After.		
2 to 4	0.44	63	3 to 4	0.34	27	3 to 3½	0.32	15	3 to 3½	0.30	8
4 " 6	0.42	47	4 " 5	0.30	26	3½ " 4	0.29	16			
6 " 8	0.30	37	5 " 6	0.26	24	4 " 5	0.27	38			
8 " 10	0.32	31	6 " 7	0.20	27	5 " 6	0.32	35			
10 " 12	0.24	15	7 " 8	0.20	20	6 " 6½	0.27	10			
						6½ " 7	0.29	19			

that the blood phosphatide increases, resulting from the ingestion of large amounts of lecithin by dogs, were in some instances more marked than the total fatty acid increases. The writer objects

to this method of studying the problem, especially since a transformation of neutral fat to lecithin is known to occur in the blood and, furthermore, for the reason that a small increase in the total fatty acid content of this *circulating fluid* may in reality be sufficient to account for a considerable transformation of ingested lecithin to neutral fat. An examination of the thoracic lymph must, therefore, be a more valid method for studying this problem. It is true that a slight increase in the organic phosphorus of the lymph occurred in these experiments. This, however, is not considered by the writer as good evidence for the view that an actual synthesis of the phosphatide had taken place, for the reason that the lecithin increments are by no means comparable to the augmentations of the fatty acid content of the lymph. This is exceptionally well emphasized in Experiment 22 (see

TABLE XI.

Maximum Increments Expressed as Percentage of the Total Fatty Acids and Lecithin in the Thoracic Lymph of Fasting Dogs.

Experiment No.	Total fatty acids.	Lecithin.
	<i>per cent</i>	<i>per cent</i>
14	851	110
20	461	60
21	242	60
23	445	50

Tables IX and X) in which unusual fluctuations in the chyle fat took place without a corresponding variation in the ether-alcohol-soluble phosphorus. A close parallelism should exist between the two factors if the evidence is to be considered favorable for the lecithin synthesis. Table XI shows to what extent the total fatty acid increments differ from the lecithin increases.

For the reasons stated above it is thought improbable that a synthesis of lecithin occurs in the intestinal mucosa; certainly here under the most ideal conditions, *i.e.* in the presence of all of the radicles required for such a synthesis, no conclusive evidence of such a transformation was secured.

In conclusion the writer wishes to call attention to the results of the control experiments summarized in Tables XII and XIII. It is quite evident that the changes in the lecithin and fat contents of the thoracic lymph caused by the anesthesia and surgical

shock are negligible, and it is, therefore, clear that the variations following the ingestion of fat, fatty acid, and lecithin are actual variations and are not due to the conditions under which the experiments were conducted.

TABLE XII.

Effect of Anesthesia on the Total Fatty Acid Content of the Thoracic Lymph of Fasting Dogs.

Time after starting anesthesia.	Chloroform. Experiment 1.		A. C. E.* Experiment 2.		A. C. E.* Experiment 3.	
	Fatty acids.	Lymph volume.	Fatty acids.	Lymph volume.	Fatty acids.	Lymph volume.
<i>hrs.</i>	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>cc.</i>
2 to 3	0.24	15	0.29	38	0.29	63
3 " 5	0.25	32	0.29	41	0.28	28
5 " 7	0.26	29	0.26	26	0.30	15
7 " 9	0.24	33				
9 " 11	0.27	21				
11 " 13	0.26	18				

* Alcohol-chloroform-ether mixture.

TABLE XIII.

Effect of Anesthesia on the Lecithin Content of the Thoracic Lymph of Fasting Dogs.

Time after starting anesthesia.	Chloroform. Experiment 1.		A. C. E.* Experiment 2.		A. C. E.* Experiment 3.	
	Lecithin.	Lymph volume.	Lecithin.	Lymph volume.	Lecithin.	Lymph volume.
<i>hrs.</i>	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>cc.</i>
2 to 3	0.20	15	0.21	38	0.21	63
3 " 5	0.25	32	0.20	41	0.20	28
5 " 7	0.18	29	0.21	26	0.20	15
7 " 9	0.20	33				
9 " 11	0.21	21				
11 " 13	0.22	18				

* Alcohol-chloroform-ether mixture.

SUMMARY.

The lecithin content of the thoracic lymph of dogs was found to be independent of the absorption of a fat (olive oil).

The ingestion of oleic acid was followed by an apparent increase in the phosphatide content of the lymph. This apparent increase does not necessarily imply a synthesis of the phosphatide in the

small intestine, for if this were true a similar augmentation should follow the ingestion of palmitic acid. Such a result was not obtained. It is, therefore, not unlikely that the lecithin increments resulting from the absorption of oleic acid may be ascribed to the known toxicity of this unsaturated fatty acid.

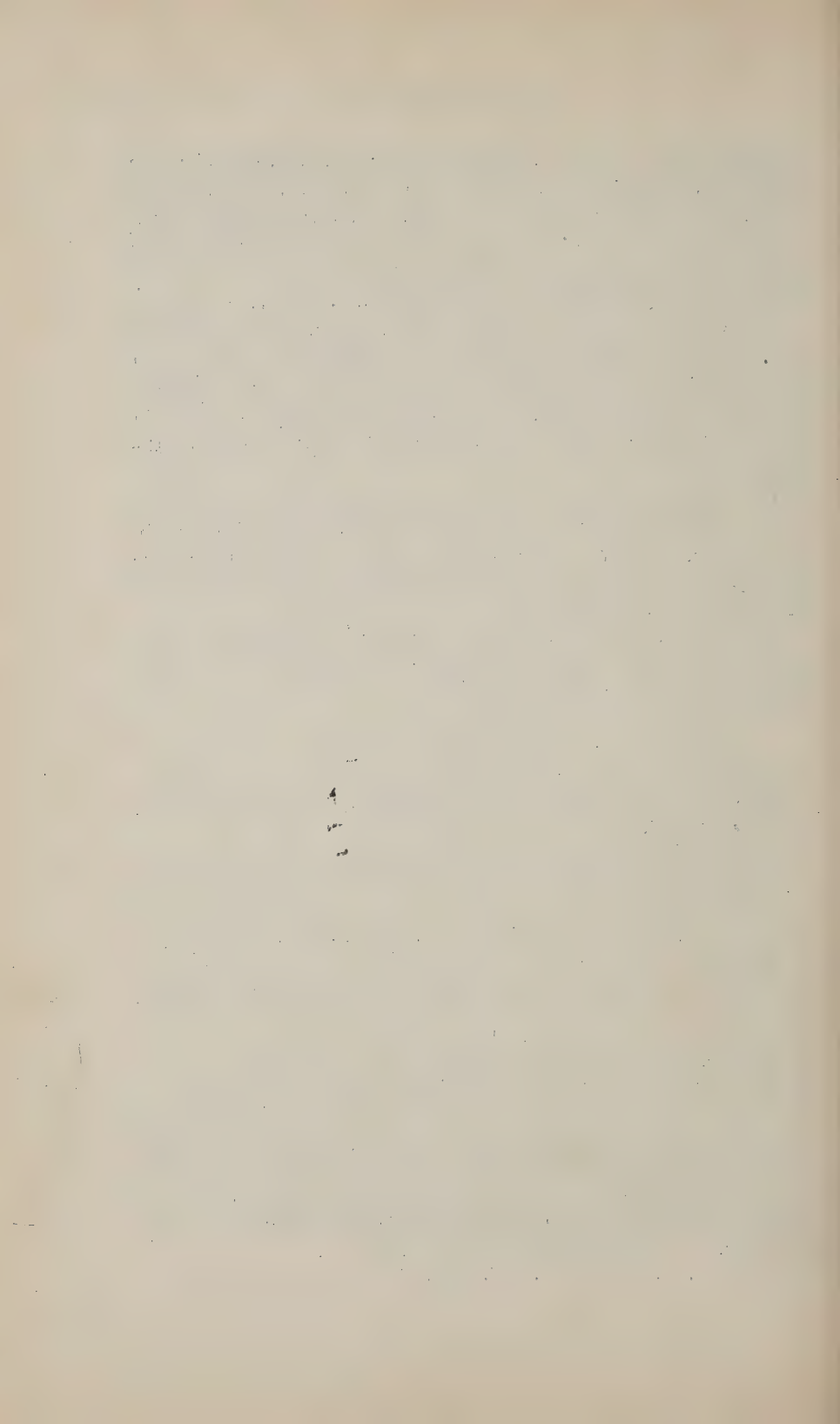
The ingestion of lecithin itself resulted in an apparent augmentation in the phosphatide content of the lymph as judged by an increase in the ether-alcohol-soluble phosphorus. This is not considered as a convincing demonstration of the case in question, since the phosphatide increments were only slight and did not parallel the fluctuations in the total fatty acid content of the lymph.

The most pronounced effect of the ingestion of lecithin is found in the increase in the total fatty acid content of the lymph. From the evidence presented it is apparent that, like simple fats, lecithin is transformed into neutral fat during its transport through the intestinal mucosa.

There is no good reason to believe that the synthesis of lecithin from ingested fat takes place prior to the entrance of the triglycerides into the blood stream.

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PLANT PHOSPHATIDES.

I. LECITHIN AND CEPHALIN OF THE SOY BEAN.

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The more modern methods of analysis of phosphatides of animal origin have not as yet been applied to the study of the phosphatides of the plant. A review of the work on this subject up to 1918 is given in the monograph by Maclean on "Lecithin and allied substances." Maclean made it clear that accurate information is lacking on every detail of the structure of plant phosphatides. It was found that in the animal tissues there are present several lecithins differing in the character of the constituent fatty acids. As regards proteins and carbohydrates, the plant kingdom shows a much greater number of variations than does the animal, and *a priori* one might possibly expect a wider variability in the composition of the plant phosphatides as compared with that of the phosphatides of animal origin.

For many reasons, the work on plant phosphatides presented great difficulties and, therefore, as far as could be ascertained, only the basic constituent choline has been identified. Neither the saturated nor the unsaturated acids were satisfactorily identified and no reliable data exist regarding the character of the glycerophosphoric acid.

Through the courtesy of Dr. H. Bollmann¹ of the *Hanseatische Mühlenwerke* a considerable quantity of a commercial lecithin obtained from soy beans was placed at our disposal. From this material lecithin was prepared which was free from cephalin. The yield of the pure product, however, was not sufficiently large to permit a complete study. In the present investigation attention was directed principally to the analysis of the fatty acids.

¹ We wish to acknowledge our indebtedness to Dr. Bollmann for supplying the material used in this investigation.

It was known from the earlier work on plant lecithin that it contained saturated and unsaturated fatty acids. It was found that soy bean lecithin contained the same two saturated fatty acids, stearic and palmitic, which occur in the lecithin of animal origin. The acids were fractionated by distillation of the methyl esters and identified in the form of the free acids. The proportion of saturated to unsaturated fatty acids seems to be smaller in the plant than in the animal lecithin and this in spite of a lower iodine value.

As regards the unsaturated fatty acids, it was definitely established that they all belong to the C_{18} series. Acids of the higher series could not be detected. Representatives of three different orders of unsaturation were found; namely, of the oleic, linolic, and linolenic types. The latter two were analyzed in the form of their bromo derivatives. The presence of oleic acid is practically certain, although as yet it has not been isolated in pure form.

In addition to the ordinary unsaturated fatty acids, a fraction was invariably found which was characterized by its insolubility in petroleic ether. The proportion of these acids was comparatively small. On the basis of analytical data this fraction seems to consist of partially hydroxylated fatty acids. They are probably secondary products.

Thus, for the present, the principal distinguishing characteristics of soy bean lecithin are: the low proportion of saturated fatty acids, the absence of unsaturated fatty acid containing a longer carbon chain than C_{18} , and the presence of linolenic acid.

In addition to pure lecithin the commercial product also contained cephalin. This phosphatide, however, when first prepared, contained many impurities. The variable composition of the products obtained in the cephalin fraction reminds one of the experience which Schulze and his coworkers had in their attempts to isolate lecithin. However, by purification from acetic acid it was possible to obtain a sample of cephalin which possessed a theoretical composition and which contained only 25 per cent of lecithin.

EXPERIMENTAL.

A. Separation of the Lecithin Fraction.—The lipoid fraction obtained from soy beans was freed from oil by thorough extraction

with acetone. The residue was fractionated with warm alcohol and from the extract the lecithin was precipitated as the cadmium chloride salt. This salt, after repeated precipitation by acetone from an ether-water solution,² contained 56 per cent of its nitrogen as amino nitrogen and had an iodine value of 20. The lecithin cadmium chloride obtained from soy beans differs from the salt prepared from animal materials in its behavior towards solvents. The two usual methods of purification used for the latter salt,² namely solution in toluene followed by precipitation with ether containing 1 per cent of water, and solution in ether containing a high percentage of water, followed by separation on cooling, are not applicable to the vegetable lipoid. The salt from soy bean lecithin is insoluble in toluene, except when highly contaminated with cephalin. While the cadmium salt is readily soluble in ether by the addition of water, on cooling such a solution, the salt separates in highly colloidal condition and very poor yield. It is possible, however, to remove the adhering cephalin by extracting the lecithin salt alternately with warm toluene and warm glacial acetic acid. A fraction purified in this manner gave the following analysis.

*Amino Distribution.*³

0.2 gm. was dissolved in 10 cc. glacial acetic acid.

5 cc. required (Kjeldahl) 4.35 cc. 0.1 N acid.

2 " : (Van Slyke) 0.04 cc. N₂ at 22°C., 756 mm.

0.2021 gm. substance absorbed 0.0639 gm. I₂.

Found.	No. 12.	$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{0.9}{100}$	Iodine value = 24.
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B. Saturated Fatty Acids of the Lecithin Fraction.—140 gm. of the lecithin cadmium chloride described above were hydrolyzed in an autoclave for 8 hours with 10 per cent HCl. By extracting the hydrolysis liquor with ether, 70 gm. of fatty acids were obtained, which had an iodine value of 102.

The mixed acids were converted into lead salts, and the unsaturated salts removed by thorough extraction with ether. The saturated fraction, freed from lead by hydrogen sulfide, yielded 10 gm. of acids melting at 56–57° C. and having a molecular weight (as indicated by titration) of 269. The low yield of the saturated

² Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv, 199.

³ Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1923, lv, 745.

acid fraction seems to be characteristic of soy bean lecithin and has been experienced in the three instances in which such lecithins have been hydrolyzed, in spite of the low iodine values of the original cadmium chloride salts.

By fractionating the methyl esters of the mixed saturated fatty acids, under diminished pressure, by the usual technique,⁴ four fractions were separated. After hydrolysis and conversion to the free acid, the lowest and highest of these gave respectively the following analyses, molecular weights, and melting points.

No. 51.	0.1006 gm. substance:	0.2768 gm. CO ₂ and 0.1142 gm. H ₂ O.
	0.8075 " " "	required for neutralization 6.25 cc. 0.5 N NaOH.
No. 52.	0.1004 gm. substance:	0.2798 gm. CO ₂ and 0.1146 gm. H ₂ O.
	0.7095 " " "	required for neutralization 5 cc. 0.5 N NaOH.
Calculated.	C ₁₆ H ₃₂ O ₂ .	C 74.92, H 12.58, Mol. wt. = 256; m.p. = 63–64°.
	C ₁₈ H ₃₆ O ₂ .	" 75.93, " 12.78, " " = 284; " = 70–71°.
Found.	No. 51.	" 75.03, " 12.78, " " = 259; " = 63–64°.
	" 52.	" 75.99, " 12.79, " " = 283; " = 70–71°.

C. Unsaturated Fatty Acids of the Lecithin Fraction.—The unsaturated lead salts, separated from the "whole fatty acid" fraction by their solubility in ether, were converted into the free acids. This fraction consisted of 30 gm. of fatty acids, having an iodine value of 131.

15 gm. of these acids were dissolved in gasoline (40–60°), filtered from a gummy gasoline-insoluble residue, and brominated below 0°C., with a 10 per cent solution of bromine in gasoline. The insoluble precipitate after standing for several days was separated by decantation (A). It was dissolved in a large volume of ether, and to this solution gasoline was added until the whole became turbid. On standing, a slight precipitate formed (B), consisting of a thick yellow oil. When treated with small quantities of cold acetone, the oil deposited crystals which, after recrystallization from benzene, melted at 180–181°C. Mixed with a sample of hexabromostearic acid obtained from linseed oil, no depression of the melting point could be observed. This sample analyzed as

⁴ Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlii, 202.

follows:

No. 47. 0.1060 gm. substance: 0.1108 gm. CO_2 and 0.0380 gm. H_2O .
 0.1067 " " : (Carius) 0.1608 gm. AgBr .
 Calculated. $\text{C}_{18}\text{H}_{30}\text{O}_2\text{Br}_6$. C 28.49, H 3.99, Br 63.26; m.p. = 180–181°.
 Found. " 28.50, " 4.01, " 64.44.

The ether-gasoline mother liquor (B), from which the above material was deposited, was concentrated to a small bulk, under diminished pressure. On standing in the ice chest 5.5 gm. of crude tetrabromostearic acid were obtained, which, after one recrystallization from ether, melted at 113°. After repeated recrystallizations from ether, the characteristic white needles, melting (with stirring) at 114°, were separated. Mixtures of this material with the tetrabromostearic acid obtained from egg lecithin⁵ and with that prepared from linolic acid, showed no depression of the melting point. This substance analyzed as follows:

No. 46. 0.1088 gm. substance: 0.1444 gm. CO_2 and 0.0538 gm. H_2O .
 0.1143 " " : (Carius) 0.1442 gm. AgBr .
 Calculated. $\text{C}_{18}\text{H}_{32}\text{O}_2\text{Br}_4$. C 36.01, H 5.38, Br 53.28.
 Found. " 36.19, " 5.53, " 54.82.

From the original bromination liquors (A) a yellow oil was isolated, which had the characteristics of dibromostearic acid. The crude material gave the following analysis.

No. 33. 0.1021 gm. substance: 16.51 gm. CO_2 and 0.0708 gm. H_2O .
 0.1152 " " : (Carius) 0.1144 gm. AgBr .
 Calculated. $\text{C}_{18}\text{H}_{34}\text{O}_2\text{Br}_2$. C 48.86, H 7.69, Br 36.18.
 Found. " 45.01, " 7.75, " 43.16.

An attempt to purify it through a fractional distillation of the methyl ester resulted in the decomposition of the material and its final identification must be deferred until further material is available.

15 gm. of the mixed unsaturated fatty acids were esterified and the methyl esters hydrogenated in the presence of palladium. The saturated esters were fractionally distilled at 1 mm., yielding three fractions and a residue. The first two fractions, aggregating 6 gm., after saponification yielded acids melting at 70–71° and 71–71.5°, respectively. They gave the following analyses.

⁵ Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 513.

No. 58.	0.0996 gm. substance:	0.2762 gm. CO ₂ and 0.1134 gm. H ₂ O.
	0.3392 " "	required for neutralization 12.1 cc. 0.1 N NaOH.
No. 59.	0.0998 gm. substance:	0.2774 gm. CO ₂ and 0.1132 gm. H ₂ O.
	0.3508 " "	required for neutralization 12.3 cc. 0.1 N NaOH.
Calculated.	C ₁₈ H ₃₆ O ₂ .	C 75.98, H 12.76, Mol. wt. = 284; m.p. = 70-71°.
Found.	No. 58.	" 75.62, " 12.94, " " = 280.
	" 59.	" 75.79, " 12.69, " " = 285.

The residue from the distillation was saponified and converted into a barium salt. This analyzed as follows:

No. 45.	0.1066 gm. substance:	0.2118 gm. CO ₂ and 0.0670 gm. H ₂ O.
	0.0971 " "	; 0.0274 " BaSO ₄ .
Calculated.	C ₁₈ H ₃₆ O ₄ · $\frac{1}{2}$ Ba (barium dihydroxystearate).	C 56.4, H 9.13, Ba 17.92.
	C ₁₈ H ₃₆ O ₅ · $\frac{1}{2}$ Ba (barium trihydroxystearate).	C 54.1, H 8.76, Ba 17.16.
	C ₂₀ H ₃₈ O ₅ · $\frac{1}{2}$ Ba (barium trihydroxyarachidate).	C 56.2, H 9.12, Ba 16.05.
Found.	C 54.18, H 7.03, Ba 16.60.	

This acid seemed to be the partly saturated derivative of the acid which occurs consistently in the unsaturated fatty acid fraction and from which it may be isolated by its insolubility in gasoline. The acid itself is a dark, gummy material, readily soluble in the usual organic solvents with the exception of gasoline. It yields well defined sodium, barium, and lead salts. One such acid after purification through its sodium and barium salts, when titrated with NaOH, seemed to have a molecular weight of 255. Although highly unsaturated (as indicated by its iodine value (70)) even as the methyl ester, this acid is very resistant to reduction with hydrogen in the presence of palladium. One such ester, after hydrogenation, was as complete as possible, was saponified, and the acid purified by conversion through the sodium and lead salts. Its physical characteristics were unchanged. It still absorbed bromine, and gave the following analysis.

No. 219.	0.1002 gm. substance:	0.2602 gm. CO ₂ and 0.0872 gm. H ₂ O.
	Found.	C 70.81, H 9.73.

D. Cephalin Fraction.—From the alcohol-insoluble residue of the lipid material, cephalin fractions were isolated by the usual

methods. These materials have as yet showed no marked differences from that isolated from animal sources.⁶ The analyses of several such samples follow.

- No. 16. 0.0990 gm. substance: 0.2232 gm. CO₂, 0.0824 gm. H₂O, and 0.0132 gm. ash.
0.1904 gm. substance required (Kjeldahl) 2.30 cc. 0.1 N acid.
0.2856 " " : (fusion) 0.0402 gm. Mg₂P₂O₇.
0.2 gm. dissolved in 10 cc. glacial acetic acid.
5 cc. required (Kjeldahl) 1.05 cc. 0.1 N acid.
2 " : (Van Slyke) 0.85 cc. N₂ at 22°C., 766 mm.
0.2914 gm. substance absorbed 0.2747 gm. I₂.
- No. 55. 0.1012 gm. substance: 0.2348 gm. CO₂, 0.0886 gm. H₂O, and 0.0104 gm. ash.
0.1917 gm. substance required (Kjeldahl) 2.25 cc. 0.1 N acid.
0.2875 " " : (fusion) 0.0424 gm. Mg₂P₂O₇.
0.2 gm. dissolved in 10 cc. glacial acetic acid.
5 cc. required (Kjeldahl) 4.20 cc. 0.1 N acid.
2 " : (Van Slyke) 2.10 cc. N₂ at 22°C., 763 mm.
0.2754 gm. substance absorbed 0.2446 gm. I₂.
- No. 67. 0.0968 gm. substance: 0.2234 gm. CO₂, 0.0816 gm. H₂O, and 0.0100 gm. ash.
0.1898 gm. substance required (Kjeldahl) 2.00 cc. 0.1 N acid.
0.2847 " " : (fusion) 0.0412 gm. Mg₂P₂O₇.
0.2 gm. dissolved in 10 cc. glacial acetic acid.
5 cc. required (Kjeldahl) 2.85 cc. 0.1 N acid.
2 " : (Van Slyke) 1.19 cc. N₂ at 22°C., 768 mm.
0.2920 gm. substance absorbed 0.2687 gm. I₂.

From No. 67, on further purification with acetic acid and alcohol, a material having the following composition was isolated.

- 0.1032 gm. substance: 0.2422 gm. CO₂, 0.0914 gm. H₂O, and 0.0102 gm. ash.
0.1951 gm. substance required (Kjeldahl) 2.40 cc. 0.1 N acid.
0.2921 " " : (fusion) 0.0404 gm. Mg₂P₂O₇.
0.2 gm. dissolved in 10 cc. glacial acetic acid.
5 cc. required (Kjeldahl) 1.10 cc. 0.1 N acid.
2 " : (Van Slyke) 0.89 cc. N₂ at 21°C., 769 mm.

⁶ Levene, P. A., and Komatsu, S., *J. Biol. Chem.*, 1919, xxxix, 91.
Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 373. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, liv, 94.

Calculated. $C_{41}H_{80}O_8$ NP (cephalin containing stearic and oleic acids).

C 65.80, H 10.77, N 1.87, P 4.15, Iodine value = 33.

$C_{41}H_{76}O_8$ NP (cephalin containing stearic and linolenic acids).

C 66.28, H 10.33, N 1.89, P 4.17, Iodine value = 99.

No. 16. Found (calculated ash-free).

C 61.48, H 9.70, N 1.69, P 3.92, Iodine value = 94.

$$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{85}{100}$$

No. 55. C 62.67, H 9.73, N 1.64, P 4.11, Iodine value = 89.

$$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{100}{100}$$

No. 67. C 64.65, H 9.52, N 1.48, P 4.03, Iodine value = 92.

$$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{97}{100}$$

No. 77. C 64.64, H 10.10, N 1.72, P 3.85.

$$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{75}{100}$$

VARIATIONS IN THE CONCENTRATION OF THE GLOBULIN AND ALBUMIN FRACTIONS OF THE BLOOD PLASMA OF YOUNG CALVES AND A COW FOLLOWING THE INJECTION OF BACILLUS ABORTUS.
VARIATIONS IN THE CONCENTRATION OF THE PROTEIN FRACTIONS OF THE BLOOD PLASMA OF PREGNANT AND NON-PREGNANT COWS OR OF COWS WHICH HAVE ABORTED.

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The relation of the changes in the protein fractions of the blood to variations in the degree of immunity of an animal is still an open question. The problem is a very complex one. In addition to the biological variables which exist in animals, an attack is hampered by deficiencies of the methods employed for the estimation of the proteins and by the uncertainties of the characterization of the protein fractions. The young calf seemed to be a particularly desirable animal to use for the study of the effect of immunization with living bacteria (*Bacillus abortus*) upon the differential distribution of the proteins of the blood plasma. The particular value of the calf lies in the fact that at about 6 weeks of age the concentrations of the various globulin fractions attain fairly definite values—which are lower than those present in the adult animal—irrespective of whether or not the calf received colostrum at birth (1). The concentration of protein in the young calf remains essentially constant for at least 6 months.

The plan of the experiment was to produce a high titer serum in the calf, by subcutaneous injections of living bacteria. If there is a direct relationship between the increase in demonstrable

antibodies and one or more of the protein fractions of the plasma this should be particularly evident because of the low "base line" of the calf. That such an increase could occur in a cow whose globulin content was unusually high had been determined in one case which will be presented later. It was also hoped that some evidence would be obtained as to the cause of the greater concentration of globulins in the cow than in the young animal—which at present can only be ascribed to age—on the hypothesis that the higher protein concentrations in the adult animal are the result of reactions to infections which have caused a greater production of proteins as immunity develops. The evidence which we have obtained, instead of answering the questions raised, presents new problems with regard to the reaction of the young animal. There was not a distinct rise in the concentration of the protein of the plasma nor was there a consistent appearance of the demonstrable evidences of immunity, as manifested by the agglutinins, complement-fixing bodies, or hemolysin (to injections of rabbit cells).

EXPERIMENTAL.

Three calves, Nos. 1079, 1080, and 1081, were obtained from a nearby dairy soon after birth and after they had remained with the mother for a few hours, in order to obtain colostrum. These calves were fed on milk obtained from cows well along in the period of lactation. At the age of 50 days Calves 1080 and 1081 were given a subcutaneous injection of 1 cc. of a suspension of *Bacillus abortus* (equal in density to a 24 hour boullion culture of *Bacillus typhosus*). Calf 1079 was kept as a control. The second injection was made 3 weeks later—Calf 1081 died the day following the injection from a cause which is unknown but which appeared not to be from the effects of the injection. The subsequent injections of Calf 1080 were made at intervals of 2 weeks, and the amount of suspension and time of injection of *Bacillus abortus* for the succeeding injections are indicated in Charts 1 and 2. Calf 1090 was introduced into the experiment to replace Calf 1081 on March 17, 1924 (41 days old, when the other calves were 114 (No. 1080) and 115 (No. 1079) days old). 1 cc. of a *Bacillus abortus* culture was given subcutaneously at this time, and the dose was gradually increased during the subsequent inoculations.

Blood was taken in all cases from the jugular vein just before the injection and again 2 days later. Determinations were made of complement-fixing bodies, of the agglutinins, and of the concentrations of the various globulin fractions of the plasma. The determination of the protein fractions was made according to the modified¹ procedure of Howe (2). The following concentrations, volume-molar, of sodium sulfate at 37°C., were used: fibrinogen, 0.75; euglobulin, 1.00; pseudoglobulin I, 1.25; and pseudoglobulin II, 1.50. The albumins were similarly fractioned on the assumption that the increment, 0.25 mol, of sodium sulfate found for the globulin fractions can be continued into the region of concentrations of salt which precipitate the albumins. The fractions are numbered as a part of a continuous series with the globulin fractions in which fibrinogen is Fraction I and pseudoglobulin II is Fraction IV. The concentrations of sodium sulfate for the albumin fractions are then V, 1.75; VI, 2.00; VII, 2.25; VIII, 2.50; and IX, 2.75.² All protein is precipitated at Fraction IX. Precipitations were made in duplicate. Because of the similarity of the variations of the protein fractions in the blood of different animals bled at the same time, which were obtained early in this work, the two precipitations made at any concentration were made with different solutions of sodium sulfate which had been prepared at different times. This precaution was taken to prevent any possibility of accidental change in the solution as it stood in the incubator.

The agglutination and complement fixation titers were determined on samples of serum obtained at the same time as the

¹ The modification refers to the slight changes in the concentrations of sodium sulfate and the use of sodium sulfate for the precipitation of fibrinogen (2).

² Fractionation of the albumin of blood. In addition to the assumption made, *i.e.* that there is a constant amount of salt to be added to that required for the precipitation of one globulin fraction in order to obtain the next fraction, we have found evidence of critical zones in the curve of precipitation of albumin, such as were found for the globulins (3). These data were obtained with plasma in which the proportion of the various fractions varied. The precipitation limits which Langstein (4) found for crystalline egg albumin when using ammonium sulfate were very sharp, and corresponded to 2.50 to 2.75 volume-molar ammonium sulfate solution or Fraction VII.

770 Globulin and Albumin Fractions of Blood

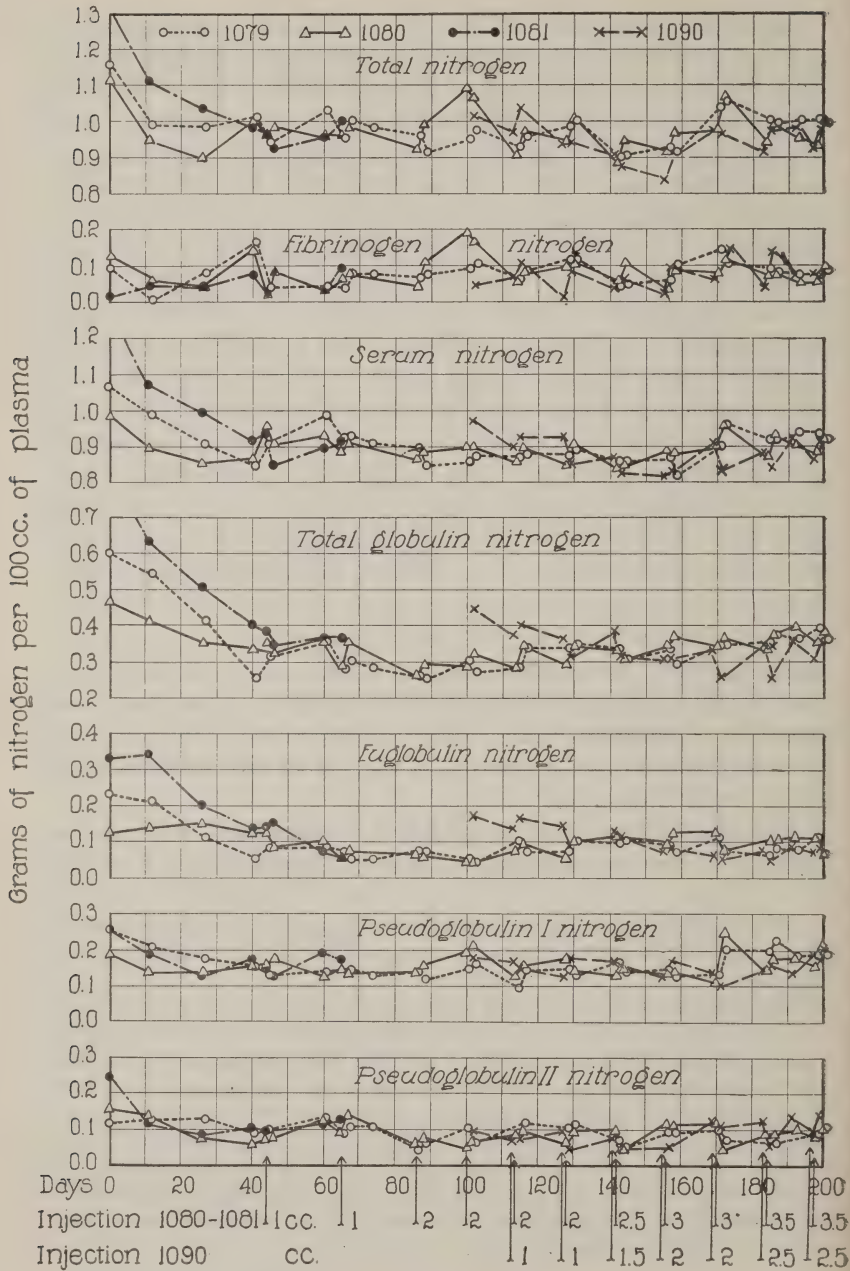


CHART 1. Protein fractions.

grams of nitrogen per 100 cc. of plasma

log of titer

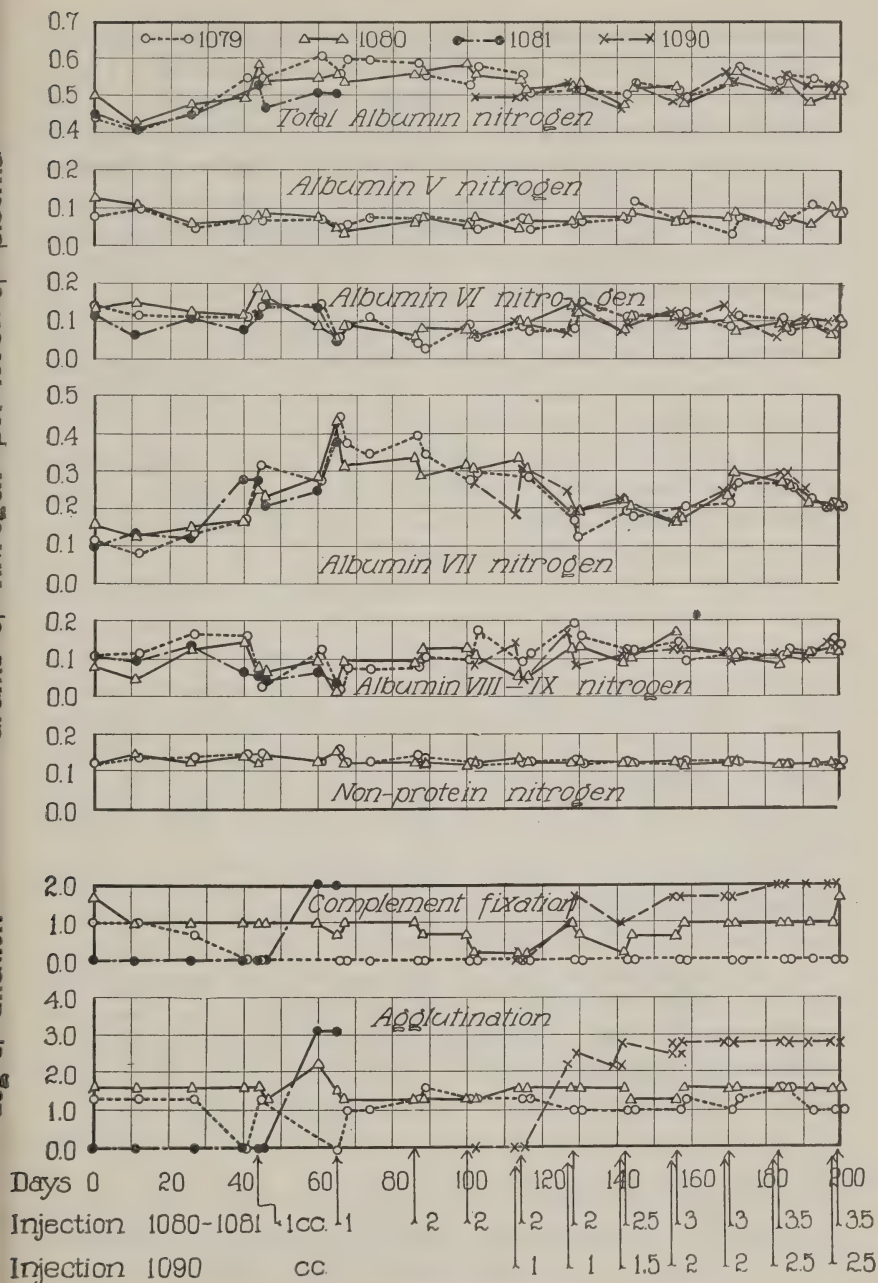


CHART 2. Protein fractions.

plasma taken for chemical analysis, using standardized procedures in conjunction with positive and negative control sera; 0.1 cc. amounts of the various serum dilutions were used in the complement fixation reaction. The results are plotted in Chart 2. The values so plotted represent the highest dilution at which an agglutination and complement fixation occurred, equivalent in most cases to a 1 plus or 2 plus on a basis in which 4 plus represented complete agglutination or complement fixation respectively. Calf 1079, the control animal, absorbed antibodies with the colostrum, and retained them, with a slight tendency to become less throughout the experiment, when judged by the agglutinin titer, or lost them completely in the course of the first 40 days according to the complement fixation reaction.

Calf 1080 absorbed antibodies from its mother's colostrum and under the influence of repeated subcutaneous injections of suspensions of living *Bacillus abortus* did not show evidence of an increased production of antibodies.

Calf 1081 did not absorb antibodies from its mother's colostrum. As the result of the first injection of *Bacillus abortus* the agglutinin titer rose from negative to 1 plus at a dilution of 1:1,280; the complement fixation reaction was 1 plus at a dilution of 1:100. This calf died suddenly the day following the second injection. The results of the autopsy did not indicate that the death was the direct result of the injection. The temperature of this calf had been slightly increased since the first injection.

Calf 1090 did not absorb antibodies at birth—a test of the blood of the mother was also negative to both the agglutination and complement fixation tests. As the result of the first injection the agglutinin titer rose from negative to 3 plus at 1:160 (\pm at 1:320). Following the second injection the titer rose to 1 plus at 1:640. Subsequent injections did not appreciably increase the titer of the serum. At the end of the experiment the titer was 3 plus at 1:640. The complement fixation test gave results which are similar to those obtained for the agglutination test.

A fifth calf (No. 1065) was injected with washed rabbit corpuscles. The blood serum was tested for hemolysin and analyzed for the changes in the concentrations of the various protein frac-

tions. The first injections of 10 cc. of a 10 per cent suspension of the red blood cells of the rabbit were made when the calf was 20, 21, and 22 days old. At this time the blood still contained large quantities of the globulins absorbed from the colostrum taken at birth. Subsequent injections of cells were made at intervals of a week as follows: 27th to 29th days, 10 cc. of a 25 per cent suspension, hemolytic titer ± 0.1 ; 34th to 36th days, 10 cc. of a 40 per cent suspension, hemolytic titer ± 0.1 ; 41st to 43rd days, 10 cc. of a 25 per cent suspension, hemolytic titer ± 0.1 ; 49th to 50th days, 10 cc. of a 20 per cent suspension, hemolytic titer ± 0.1 ; 55th day, 10 cc. of a 35 per cent suspension, hemolytic titer ± 0.1 .³

The data relating to the variations in the concentrations of protein are contained in Tables I to IV and Charts 1 and 4. The results on Calf 1090 are plotted in relation to the time of the bleeding of Calves 1079 and 1080. The ordinate representing the birth of the calf is, therefore, at 60 days and the other values must be considered accordingly.

DISCUSSION.

Effect of the Subcutaneous Inoculation with Living Bacillus abortus or Rabbit Corpuscles on Immune Reactions.—Four cases have been studied. One was a control, three were injected with *Bacillus abortus* and one with rabbit corpuscles. Two calves, Nos. 1080 and 1065, did not produce detectable antibodies in any appreciable amount. The control calf (No. 1079) had a titer of 1:20 for *Bacillus abortus* as the result of the absorption of colostrum, which persisted with slight fluctuations until the end of the experiment.

Two other calves, Nos. 1081 and 1090, whose blood was negative or low in antibodies for the *Bacillus abortus* after suckling, developed a fairly low titer with the first injection, but did not show any particular increase as the result of subsequent injections of *Bacillus abortus*.

³ The injections and the determinations of the hemolytic titers were made by Dr. F. S. Jones in the course of some work on which he was engaged. We are indebted to him for the opportunity of analyzing the serum and for the results of his determinations.

TABLE I.
Results Obtained from the Analysis of the Blood Plasma of Calf 1079 (Control Animal).

Days.	Total N.	Serum N.	Fibrinogen N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.*	Albumin N.					Non-protein N.
					I	II		V	VI	VII	VIII-IX	Total.	
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
1	1.160	1.064	0.096	0.226	0.251	0.113	0.600	0.078	0.140	0.115	0.106	0.439	0.025
12	0.993	0.986	0.007	0.214	0.205	0.128	0.548	0.098	0.111	0.082	0.111	0.402	0.037
17	0.988	0.906	0.082	0.113	0.175	0.129	0.417	0.045	0.111	0.132	0.164	0.452	0.037
41	1.012	0.844	0.168	0.053	0.151	0.090	0.257	0.069	0.107	0.173	0.160	0.546	0.041
45	0.944	0.904	0.040	0.084	0.128	0.100	0.312	0.066	0.140	0.316	0.021	0.543	0.049
61	1.032	0.988	0.044	0.089	0.137	0.131	0.357	0.068	0.147	0.274	0.124	0.604	0.027
66	0.958	0.922	0.036	0.072	0.137	0.090	0.288	0.049	0.057	0.433	0.017	0.556	0.053
68	1.002	0.926	0.076	0.053	0.141	0.109	0.303	0.055	0.090	0.376	0.077	0.598	0.025
74	0.983	0.907	0.076	0.051	0.126	0.109	0.286	0.073	0.111	0.347	0.075	0.596	0.025
87	0.961	0.895	0.066	0.077	0.139	0.045	0.261	0.070	0.041	0.396	0.078	0.585	0.049
89	0.916	0.844	0.074	0.075	0.119	0.061	0.255	0.074	0.025	0.347	0.106	0.552	0.037
101	0.948	0.858	0.090	0.053	0.147	0.102	0.302	0.060	0.091	0.276	0.100	0.527	0.029
103	0.979	0.871	0.108	0.045	0.160	0.069	0.274	0.041	0.057	0.299	0.180	0.577	0.020
115	0.930	0.868	0.062	0.104	0.091	0.092	0.287	0.075	0.087	0.303	0.091	0.556	0.025
117	0.960	0.876	0.084	0.075	0.141	0.120	0.336	0.042	0.075	0.283	0.111	0.507	0.029
129	0.987	0.872	0.115	0.075	0.149	0.108	0.333	0.063	0.083	0.170	0.199	0.515	0.025
131	1.002	0.884	0.118	0.104	0.128	0.116	0.348	0.067	0.158	0.128	0.161	0.515	0.021
143	0.900	0.859	0.041	0.099	0.162	0.071	0.332	0.070	0.112	0.196	0.124	0.502	0.025
145	0.906	0.859	0.047	0.106	0.137	0.054	0.307	0.116	0.115	0.180	0.121	0.532	0.020
157	0.928	0.868	0.060	0.092	0.143	0.097	0.332	0.064	0.120	0.187	0.145	0.516	0.020
159	0.914	0.814	0.100	0.071	0.125	0.095	0.291	0.066	0.126	0.204	0.098	0.494	0.029
171	1.040	0.898	0.142	0.113	0.128	0.100	0.341	0.029	0.088	0.211	0.104	0.532	0.025
173	1.058	0.951	0.107	0.079	0.200	0.071	0.349	0.075	0.116	0.270	0.116	0.577	0.025
185	1.001	0.911	0.090	0.065	0.197	0.089	0.351	0.054	0.110	0.269	0.107	0.540	0.020
187	0.999	0.915	0.084	0.082	0.225	0.065	0.372	0.069	0.074	0.253	0.127	0.552	0.020
193	1.003	0.931	0.072	0.080	0.181	0.102	0.363	0.106	0.094	0.225	0.119	0.544	0.024
199	1.005	0.931	0.074	0.114	0.188	0.090	0.392	0.086	0.065	0.212	0.156	0.519	0.020
201	0.995	0.911	0.084	0.065	0.188	0.107	0.360	0.089	0.094	0.205	0.139	0.527	0.024

Days.	Total N.	Serum N.	Fibrinogen N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.				Non-protein N.	
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	I	II	gm. per 100 cc.	V	VI	VII	VIII-IX		Total.
1	1.113	0.986	0.127	0.127	0.185	0.156	0.468	0.122	0.131	0.156	0.079	0.498	0.020
11	0.950	0.892	0.058	0.140	0.136	0.139	0.415	0.017	0.148	0.128	0.045	0.428	0.049
20	0.900	0.852	0.048	0.156	0.133	0.074	0.353	0.056	0.123	0.150	0.123	0.474	0.025
40	1.007	0.865	0.142	0.125	0.153	0.055	0.333	0.064	0.115	0.168	0.144	0.491	0.041
44	0.968	0.953	0.015	0.125	0.156	0.070	0.351	0.076	0.193	0.251	0.062	0.582	0.020
46	0.989	0.902	0.087	0.084	0.171	0.072	0.307	0.082	0.168	0.231	0.053	0.534	0.041
60	0.955	0.924	0.031	0.102	0.126	0.127	0.356	0.073	0.090	0.286	0.092	0.541	0.027
65	0.968	0.897	0.069	0.059	0.141	0.090	0.290	0.049	0.057	0.433	0.017	0.556	0.053
67	0.985	0.908	0.077	0.078	0.136	0.140	0.354	0.031	0.090	0.319	0.094	0.534	0.020
80	0.924	0.862	0.042	0.065	0.135	0.065	0.261	0.065	0.062	0.339	0.090	0.556	0.045
88	0.991	0.883	0.108	0.061	0.156	0.077	0.294	0.074	0.082	0.286	0.122	0.564	0.025
100	1.093	0.899	0.194	0.049	0.192	0.049	0.290	0.053	0.082	0.319	0.130	0.584	0.025
102	1.067	0.899	0.164	0.045	0.212	0.066	0.323	0.077	0.062	0.308	0.109	0.556	0.020
114	0.909	0.855	0.054	0.075	0.124	0.087	0.286	0.046	0.104	0.333	0.056	0.540	0.029
116	0.973	0.893	0.080	0.092	0.153	0.095	0.341	0.062	0.100	0.303	0.054	0.519	0.033
128	0.946	0.847	0.099	0.058	0.175	0.062	0.295	0.066	0.146	0.191	0.124	0.527	0.025
130	1.038	0.901	0.107	0.104	0.141	0.100	0.345	0.074	0.129	0.194	0.134	0.531	0.025
142	0.889	0.834	0.055	0.117	0.125	0.100	0.332	0.079	0.078	0.225	0.095	0.477	0.025
144	0.949	0.843	0.106	0.116	0.142	0.049	0.307	0.088	0.112	0.203	0.109	0.512	0.024
156	0.916	0.885	0.031	0.092	0.130	0.119	0.341	0.067	0.116	0.167	0.174	0.524	0.020
158	0.965	0.876	0.089	0.125	0.133	0.112	0.370	0.079	0.096	0.174	0.131	0.481	0.025
170	0.978	0.898	0.080	0.121	0.108	0.116	0.345	0.071	0.108	0.237	0.116	0.523	0.021
172	1.070	0.959	0.111	0.079	0.249	0.045	0.369	0.084	0.074	0.299	0.108	0.565	0.025
184	0.942	0.870	0.072	0.102	0.143	0.086	0.331	0.057	0.098	0.270	0.089	0.514	0.025
186	0.999	0.927	0.072	0.110	0.172	0.090	0.372	0.073	0.082	0.265	0.115	0.535	0.020
192	0.951	0.899	0.052	0.115	0.179	0.103	0.397	0.057	0.094	0.216	0.115	0.482	0.020
198	0.932	0.878	0.054	0.110	0.155	0.086	0.351	0.102	0.066	0.212	0.123	0.503	0.024
200	1.009	0.911	0.098	0.065	0.213	0.102	0.380	0.086	0.102	0.200	0.123	0.511	0.020

TABLE III.
Results Obtained from the Analysis of the Blood Plasma of Calves 1090 and 1081.

Days.	Total N.	Serum N.	Fibri- nogen N.	Euglob- ulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.					Non-pro- tein N.
					I	II		V	VI	VII	VIII-IX	Total.	

Calf 1090.													
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
30	1.108	0.968	0.049	0.172	0.176	0.098	0.446	0.082	0.061	0.266	0.089	0.498	0.025
41	0.968	0.897	0.072	0.137	0.167	0.070	0.374	0.066	0.100	0.187	0.145	0.498	0.025
43	1.040	0.926	0.106	0.170	0.148	0.077	0.405	0.062	0.091	0.303	0.042	0.498	0.033
55	0.938	0.926	0.015	0.143	0.124	0.096	0.363	0.054	0.070	0.241	0.170	0.535	0.025
57	0.938	0.855	0.083	0.091	0.175	0.045	0.311	0.096	0.145	0.191	0.087	0.519	0.025
69	0.907	0.868	0.039	0.137	0.166	0.079	0.382	0.050	0.071	0.228	0.112	0.461	0.025
71	0.879	0.826	0.053	0.104	0.157	0.051	0.311	0.054	0.092	0.218	0.117	0.491	0.024
83	0.839	0.814	0.025	0.075	0.121	0.113	0.309	0.057	0.127	0.163	0.128	0.485	0.020
85	0.914	0.822	0.092	0.087	0.166	0.055	0.308	0.077	0.101	0.192	0.124	0.494	0.020
97	0.975	0.909	0.066	0.065	0.133	0.121	0.332	0.050	0.141	0.249	0.125	0.565	0.025
99	0.969	0.822	0.147	0.054	0.100	0.108	0.262	0.066	0.120	0.254	0.095	0.535	0.025
111	0.914	0.874	0.040	0.078	0.143	0.126	0.347	0.037	0.057	0.298	0.115	0.507	0.020
113	0.975	0.837	0.138	0.045	0.155	0.057	0.257	0.070	0.085	0.294	0.106	0.555	0.025
119	0.986	0.907	0.079	0.086	0.135	0.135	0.356	0.065	0.106	0.257	0.099	0.527	0.024
125	0.929	0.854	0.075	0.074	0.135	0.098	0.307	0.081	0.098	0.205	0.143	0.527	0.020
127	0.980	0.907	0.072	0.082	0.131	0.147	0.360	0.065	0.114	0.203	0.139	0.527	0.020

Calf 1081.													
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
1	1.308	1.290	0.018	0.333	0.250	0.243	0.826	0.139	0.107	0.099	0.103	0.448	0.016
11	1.113	1.072	0.141	0.341	0.189	0.107	0.637	0.113	0.061	0.132	0.094	0.410	0.025
26	1.039	0.994	0.045	0.203	0.228	0.088	0.509	0.085	0.108	0.124	0.131	0.448	0.037
40	0.982	0.916	0.076	0.133	0.171	0.101	0.405	0.092	0.077	0.279	0.064	0.495	0.016
44	0.960	0.933	0.027	0.144	0.146	0.097	0.387	0.073	0.118	0.275	0.055	0.521	0.025
46	0.929	0.846	0.083	0.152	0.123	0.074	0.349	0.073	0.148	0.206	0.041	0.469	0.028
60	0.959	0.897	0.039	0.074	0.190	0.121	0.362	0.053	0.139	0.249	0.067	0.508	0.027
61	1.005	0.912	0.093	0.062	0.174	0.128	0.364	0.037	0.049	0.380	0.037	0.503	0.045

TABLE IV.
Comparison of the Averages of Results Obtained on the Distribution of Protein in Blood Plasma Taken Just before Inoculation with Those on Blood Taken 2 Days after Inoculation.

Calf No.		Total N.	Serum N.	Fibrinogen N.	Euglobulin N.	Pseudoglobulin N.		Albumin N.		
						I	II	VI	VII	Total.
		gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
1079 Control.	1st bleeding.	0.966	0.888	0.078	0.086	0.148	0.088	0.085	0.265	0.535
	2nd " 2 days later.	0.973	0.885	0.088	0.076	0.156	0.087	0.093	0.255	0.543
1080 Injected.	1st bleeding.	0.951	0.871	0.061	0.088	0.144	0.084	0.101	0.271	0.536
	2nd "	1.000	0.900	0.100	0.087	0.170	0.086	0.100	0.253	0.531
1090 Injected.	1st bleeding.	0.924	0.878	0.047	0.101	0.141	0.100	0.095	0.224	0.510
	2nd "	0.956	0.856	0.099	0.090	0.147	0.077	0.107	0.237	0.517

The reaction to injection is not clear. It would appear from the results presented that those calves which absorbed antibodies to *Bacillus abortus* with the colostrum did not show a demonstrable increase in antibody production following subcutaneous injections of living cultures of *Bacillus abortus*. On the other hand, the calves which had not absorbed antibodies to *Bacillus abortus* gave a demonstrable increase of antibodies to the first injection, but failed to show further increase upon subsequent injections. It will require further work to determine the conditions under which the young animal develops detectable antibodies following the injection of an antigen.

Moll (5) and Famulener (6) have found it difficult to obtain hemolysin in young rabbits and goats respectively, and Schkarin (7) did not find antibodies in the blood of young rabbits following the injection of milk until after 6 weeks of age.

Rabagliati (8) reports an extended review of investigations made with regard to the immunity and reaction to inoculation of calves born of cows immunized or not immunized against cattle plague. Calves of cows immunized by double inoculation of virulent blood and of serum may or may not be resistant to a subsequent injection of virulent blood. Seven calves were tested when 1 month old and again at 5 months of age—1 month after weaning. None gave a reaction at both tests; one was positive at the first test and negative at the second; two were negative at the first test and gave a reaction at the second; and four were negative at both tests. Two calves were inoculated at birth, while their mothers were still reacting to immunization. One gave a slight reaction and the other was negative at birth; upon subsequent test both were negative when 5 months old and later when 1 year and 10 months old. Similar results were obtained with calves from cows immunized before conception. Of eight calves one reacted when 23 days old and was immune at $11\frac{1}{2}$ months, while the remaining seven were negative at from 11 to 47 days and reacted when tested later at $7\frac{1}{2}$ to 12 months. The general trend of all of the results is in the direction of an acquired resistance from the mother such that inoculation of virulent blood at approximately 1 month is negative, but is positive at the second inoculation at from 5 to 12 months, whereas a positive reaction, while still young, results in an im-

munity which protects the animal against a later infection. On the other hand, four cases were observed, as noted above, in which the calves from immune mothers did not react at any time to a subsequent injection of virulent blood. The double inoculation of calves when 12 to 18 months old confers a lasting immunity. These observations, while not entirely comparable to our experiment, indicate that the immunization of young animals may be variable.

Changes in the Plasma Proteins as the Result of the Subcutaneous Injection with Living Cultures of Bacillus abortus or with Red Blood Cells.—The permanent effect of the subcutaneous injection of living cultures of *Bacillus abortus* or of suspensions of red blood cells upon the quantity and distribution of the proteins of the blood was practically negligible.

There are three evident factors which affected the composition of the blood, in addition to those of age and the ingestion of colostrum, which have already been described (1, 9); *i.e.*, the increase and gradual decrease in globulin and the increase in albumin.

a. Withdrawal of Blood.—The taking of approximately 40 cc. of blood at each bleeding has an effect on the subsequent distribution of protein. This change is evident from the breaks in the curves in Charts 1 and 2. It is most pronounced in the case of the injected animals (Nos. 1080 and 1090), but is, nevertheless, present in the case of the control animal. In Table IV are presented data obtained by averaging the results from the analysis of the blood taken immediately before injection (12 days after the last bleeding) and those on blood taken 2 days after the injection.

The effect of bleeding is not so evident in the average data for Calf 1079 as it is in the charts because the effect was sometimes in one direction and sometimes in another.

b. Temporary Effect of Inoculation.—This change is most pronounced in the increase in fibrinogen in the injected calves accompanying the increase in temperature following the injection of the calves. This finding agrees with the results of Foster and Whipple (10) in which they found that tissue injury and inflammation exercise a powerful stimulus upon fibrinogen production. There is an individual effect in the changes of the remaining pro-

tein fractions represented by an increase in total globulin (which does not include fibrinogen) in the case of Calf 1080 and a decrease for Calf 1090.

c. Response to a Change in Environment.—There is a gradual change in the relative distribution of the protein fractions which we have interpreted as representing an effect of environment. The effect is most pronounced in the variations of Albumin Fraction VII; this fraction is the one which has the same precipitation limits as the crystalline egg albumin studied by Langstein (4). In this case there occurred during the period of investigation a rise followed by a fall, then a second rise, not so great as the first rise; a fall was in progress at the time the experiment closed. These increases agree roughly with the period of

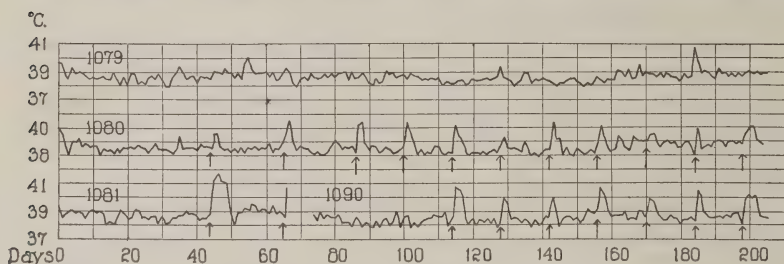


CHART 3. Temperature changes.

extreme cold in the winter and the cold spring, but we are not ready to give this as the final explanation.

The results on Calf 1090 are interesting. In Charts 1 to 4 the data for the calf are plotted with relation to the time of bleeding of Calves 1079 and 1080. When so plotted the data on total albumin and the albumin fractions fall very close to those of Calves 1079 and 1080. Had the results been plotted according to age, laid off for the first two calves, the curves would have been widely divergent. There is one objection which could be raised against the observation just made, that of experimental error. It was to meet this objection that we took unusual pains with our solutions as indicated in presenting the data. Furthermore, analyses were made of other samples of blood at the same time which gave different distributions of the protein fractions. Attention is called to an apparently independent

course in the pseudoglobulin I fraction in the blood of Calf 1090; this change, with the more or less independent change in the concentration of total globulin and the fairly constant quantity of total albumin, suggests that an environmental factor was affecting chiefly the relative distribution of the albumin fractions.

Moll (5) studied the change in globulin and albumin while immunizing young rabbits (18 to 21 days old) to foreign protein. He found an increase in globulin with the appearance of antibodies but not so great as with the adult animal. Killed cultures of the cholera bacillus caused young rabbits 3 weeks old to develop antibodies, but to a less degree than the adult. No attempt was made to study the living organism, although Moll

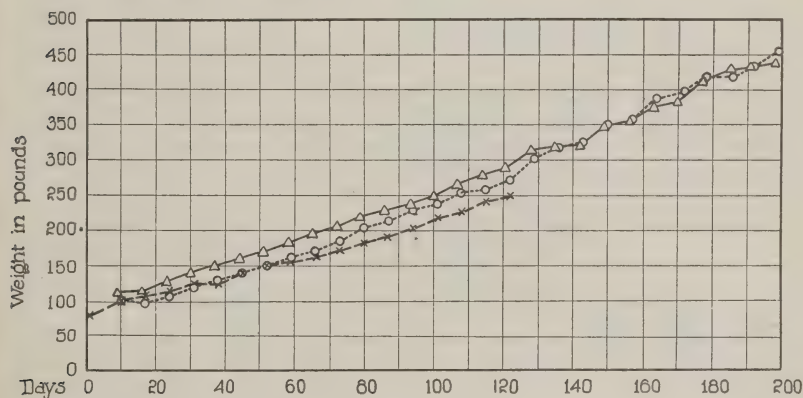


CHART 4. Body weight changes.

assumes that there would be a more marked reaction in such a case.

Effect of the Intravenous and Subcutaneous Injections of Bacillus abortus into a Cow.—During the course of the immunization of a cow for the purpose of obtaining immune serum we had an opportunity to examine the blood. The data are summarized in Chart 5. This is a purely isolated case but it has certain points which are interesting. (a) There is a very marked increase in globulin as the result of injection of the living culture, without such marked variations in the albumin, which shows a slight drop. (b) The change noted in (a) occurred in an animal whose blood was already unusually rich in globulin. (c) The degree of immunity

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is not necessarily directly related to the increase of globulin above the value obtained before immunization began—a well known fact.

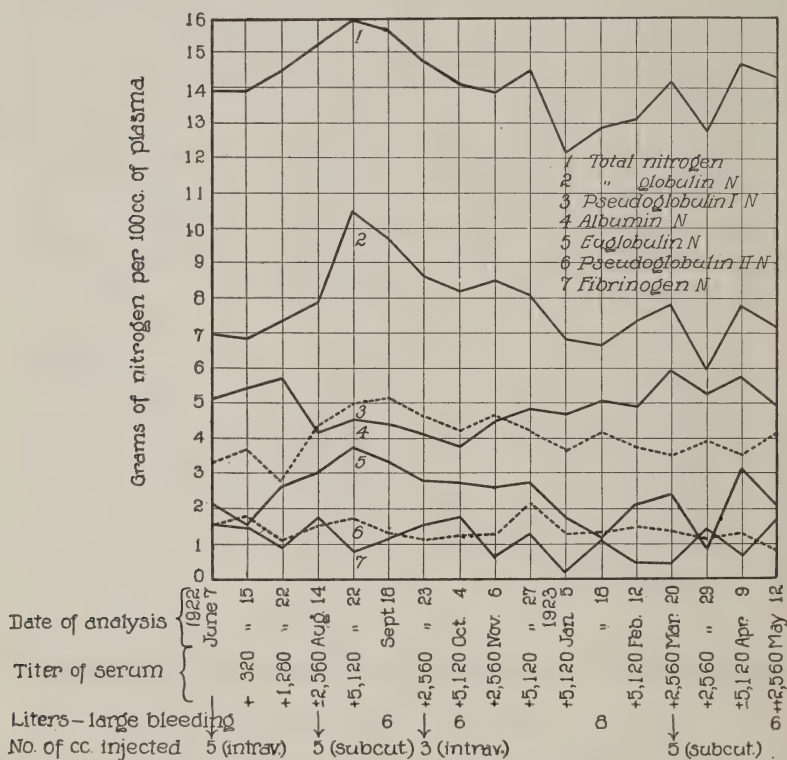


CHART 5. Immunization of a cow.

Variations in the Concentrations of the Proteins of Cows during Pregnancy and with Age.

In presenting data with regard to the variation in the concentration of various protein fractions in the blood of young calves with age (1), data were given on the composition of the blood of young non-pregnant and pregnant heifers. The cows, from which the samples were taken, were subjects of an experiment on vaccination against *Bacillus abortus*, conducted by Smith and Little (11). At various times since the original bleedings addi-

tional determinations of the proteins of the blood of certain of these cows have been made. Tables V to VII contain data on cows which calved normally, grouped according to the par-

TABLE V.

Data Showing the Distribution of the Various Protein Fractions in the Blood Plasma of Cows Which Calved Normally at Different Ages. Control Animals (Smith and Little).

Cow No.	Age.		Total N.	Serum N.	Fibri-nogen N.	Euglob-ulin N.	Pseudoglob-ulin N.		Total globulin N.	Total albu-min N.	Non-protein N.
	Bled.	Calved.					I	II			
1st bleeding.											
	days	days	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
907	667		1.184	1.084	0.100	0.036	0.293	0.136	0.464	0.557	0.062
912	631		1.175	1.035	0.140	0.078	0.279	0.138	0.495	0.490	0.050
914	612		1.316	1.114	0.202	0.042	0.375	0.140	0.557	0.499	0.058
916	604		1.305	1.126	0.179	0.042	0.395	0.157	0.594	0.462	0.056
Average..	628		1.245	1.090	0.155	0.049	0.336	0.143	0.528	0.502	0.057
2nd bleeding.											
907	899	985	1.017	1.007	0.010	0.132	0.192	0.145	0.469	0.495	0.043
912	862	951	1.019	0.999	0.020	0.107	0.214	0.085	0.406	0.513	0.080
914	840	918	1.087	1.063	0.024	0.116	0.294	0.107	0.517	0.475	0.068
916	833	1,042	1.083	1.075	0.008	0.077	0.285	0.120	0.482	0.533	0.060
Average..	865	974	1.051	1.036	0.015	0.110	0.264	0.114	0.488	0.505	0.063
3rd bleeding.											
907	1,264	1,385	1.115	1.035	0.080	0.164	0.275	0.070	0.509	0.497	0.029
912	1,222	1,288	1.096	1.027	0.069	0.078	0.201	0.181	0.460	0.538	0.029
914	1,295	1,269	1.216	1.153	0.063	0.168	0.327	0.065	0.560	0.564	0.029
916	1,202	1,370	1.054	1.007	0.047	0.074	0.304	0.042	0.420	0.546	0.041
Average..	1,263	1,328	1.120	1.055	0.065	0.121	0.279	0.089	0.487	0.536	0.030

ticular treatment they received. In Tables VIII and IX are given data on individual cows before and after abortion and on cows at the time of parturition; other data on the latter cows can be found in the preceding tables. In the tables the data presented

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are those obtained when the cow was bled at the different age groups; in many cases we have supplementary data.

TABLE VI.

Data Showing the Distribution of the Various Protein Fractions in the Blood Plasma of Cows Which Calved Normally at Different Ages. Injected with Living Vaccine (Smith and Little).

Cow No.	Age.		Total N.	Serum N.	Fibri- nogen N.	Euglob- ulin N.	Pseudoglob- ulin N.		Total globu- lin N.	Total albu- min N.	Non- pro- tein N.
	Bled.	Calved.					I	II			
1st bleeding.											
	days	days	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
928	548		1.219	1.043	0.176	0.115	0.260	0.120	0.495	0.478	0.070
920	584		1.082	1.002	0.080	0.062	0.231	0.136	0.429	0.515	0.058
925	557		1.060	0.978	0.082	0.034	0.235	0.094	0.363	0.545	0.070
Average..	563		1.120	1.008	0.112	0.070	0.242	0.117	0.429	0.513	0.066
2nd bleeding.											
928	779	932	1.075	1.050	0.025	0.145	0.252	0.103	0.500	0.482	0.068
920	815	951	1.034	0.999	0.035	0.090	0.290	0.103	0.485	0.448	0.068
925	812	922	1.108	1.084	0.024	0.051	0.286	0.094	0.431	0.589	0.064
Average..	802	935	1.072	1.044	0.026	0.095	0.276	0.100	0.471	0.506	0.067
3rd bleeding.											
928	883	932	1.053	0.980	0.073	0.121	0.209	0.122	0.452	0.484	0.044
920	919	951	1.021	1.001	0.020	0.097	0.259	0.115	0.471	0.486	0.044
925	925	922	1.157	1.109	0.048	0.106	0.259	0.073	0.438	0.582	0.089
Average..	909	935	1.077	1.031	0.047	0.108	0.242	0.103	0.453	0.517	0.059
4th bleeding.											
928	1,155	1,293	1.213	1.138	0.075	0.226	0.205	0.148	0.579	0.530	0.029
920	1,184	1,376	1.074	1.003	0.071	0.050	0.275	0.115	0.440	0.538	0.025
925	1,157	1,309	1.070	0.912	0.158	0.078	0.144	0.070	0.292	0.591	0.029
Average..	1,162	1,329	1.119	1.018	0.101	0.118	0.208	0.111	0.437	0.553	0.028

The results contained in Tables V to VII do not present any striking variations. They indicate in general that pregnancy

TABLE VII.

Data Showing the Distribution of the Various Protein Fractions in the Blood Plasma of Cows Which Calved Normally at Different Ages. Injected with Heated Vaccine (Smith and Little).

Cow No.	Age.		Total N.	Serum N.	Fibri- nogen N.	Euglob- ulin N.	Pseudoglob- ulin N.		Total globu- lin N.	Total albu- min N.	Non- pro- tein N.
	Bled.	Calved.					I	II			
1st bleeding.											
	days	days	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
722	878		1.052	0.964	0.088	0.061	0.214	0.136	0.411	0.491	0.062
725	882		1.043	0.957	0.086	0.116	0.251	0.136	0.503	0.396	0.058
726	888		1.129	1.070	0.059	0.101	0.268	0.120	0.489	0.523	0.058
729	893		1.085	1.033	0.052	0.072	0.297	0.148	0.517	0.458	0.058
734	865		1.150	1.070	0.080	0.196	0.288	0.096	0.580	0.424	0.066
749	838		1.133	1.074	0.059	0.158	0.207	0.144	0.509	0.499	0.066
740	837		1.163	1.113	0.050	0.189	0.285	0.136	0.610	0.437	0.066
743	869		1.223	1.136	0.089	0.092	0.364	0.140	0.596	0.478	0.062
739	865		1.090	1.021	0.069	0.130	0.177	0.161	0.468	0.487	0.066
Average..	868		1.118	1.048	0.070	0.124	0.261	0.135	0.520	0.465	0.063
2nd bleeding.											
722	1,092	1,100	1.058	0.973	0.085	0.047	0.260	0.098	0.405	0.512	0.056
725	1,091	1,101	0.936	0.936		0.108	0.231	0.081	0.420	0.441	0.075
726	1,084	1,100	1.210	1.118	0.092	0.128	0.269	0.133	0.530	0.545	0.043
729	1,060	1,120	1.092	1.011	0.082	0.094	0.262	0.088	0.444	0.533	0.034
734	1,095	1,090	1.277	1.067	0.210	0.167	0.187	0.193	0.547	0.503	0.017
749	1,023	1,087	0.986	0.917	0.079	0.043	0.254	0.093	0.390	0.467	0.060
739	1,049	1,088	1.101	1.051	0.050	0.119	0.247	0.103	0.469	0.531	0.051
740	1,056	1,081	1.119	1.054	0.065	0.096	0.263	0.128	0.487	0.520	0.047
743	1,154	1,245	1.096	1.061	0.035	0.097	0.246	0.101	0.444	0.565	0.052
Average..	1,077	1,112	1.097	1.021	0.077	0.099	0.246	0.113	0.458	0.513	0.048
3rd bleeding.											
722	1,460	1,511	1.085	1.040	0.045	0.136	0.214	0.111	0.461	0.546	0.033
725	1,433	1,433	1.485	1.294	0.195	0.139	0.494	0.037	0.670	0.595	0.029
726	1,460		1.254	1.171	0.083	0.119	0.391	0.148	0.658	0.488	0.025
729	1,446	1,612	1.119	1.101	0.018	0.127	0.288	0.136	0.551	0.427	0.033
734	1,441	1,433	1.022	0.949	0.073	0.057	0.190	0.114	0.361	0.559	0.029
749	1,410	1,350	1.137	1.080	0.057	0.225	0.169	0.168	0.562	0.493	0.025
739	1,435		1.296	1.212	0.084	0.255	0.304	0.090	0.649	0.530	0.033
740	1,415	1,400	1.210	1.142	0.068	0.123	0.288	0.131	0.542	0.571	0.029
743	1,397	1,586	1.250	1.187	0.063	0.201	0.312	0.058	0.571	0.579	0.037
Average..	1,433	1,475	1.206	1.130	0.076	0.154	0.294	0.110	0.558	0.532	0.030

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in the cow does not cause any marked uniform change in the distribution of the various protein fractions of the blood plasma, a fact which was found for the cow in the presentation of the first analyses on these animals. Such variations as occur are probably the result of "normal" changes which take place in the blood of a cow in which minor infections, age, or environmental conditions may play a part. A comparison of the different groups of cows does not yield evidence of the effect of immunization on the distribution of the protein fractions.

The results presented in Table IX indicate a definite change in the distribution of the protein fractions in cows which had re-

TABLE VIII.

Data Showing the Distribution of the Various Protein Fractions in the Blood Plasma of Cows at the Time of Calving.

Cow No.	Age.		Total N.	Serum N.	Fibrinogen N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Total albumin N.	Non-protein N.
	Bled.	Calved.					I	II			
	days	days	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
927	938	936		1.184		0.130	0.242	0.220	0.593	0.540	0.051
928	932	932	1.282	1.118	0.164	0.102	0.340	0.119	0.561	0.506	0.051
930	871	867	1.213	1.160	0.053	0.153	0.319	0.089	0.561	0.523	0.076
920	951	951	1.164	1.096	0.068	0.051	0.293	0.093	0.437	0.570	0.089
925	1,013	1,010	1.144	1.000	0.154	0.030	0.264	0.137	0.431	0.533	0.026
929	1,121	1,120	1.157	1.109	0.048	0.106	0.259	0.073	0.438	0.583	0.089
Average..			1.192	1.111	0.097	0.095	0.286	0.122	0.503	0.542	0.064

cently aborted. There is an average increase in fibrinogen, although certain of the individual animals showed an actual decrease; euglobulin is increased in each case; pseudoglobulin I is increased in four out of six cases. The average for pseudoglobulin II shows an increase which is due to changes which occurred in three out of the six cases. The total globulin is increased in every case. The albumin was decreased in three out of six cases. It is evident, in comparison with the data on the cows who calved normally, that with active infection there is in general an increase in globulin and a tendency for a slight decrease in the albumin fractions. These results are exclusive of

the fibrinogen which is known often to increase in the case of an infection and appears to fluctuate rather independently of the other protein fractions.

TABLE IX.

Data Showing the Distribution of the Protein Fractions of Blood Plasma before and after Abortion or Normal Parturition.

Cow No.	Age.		Total N.	Serum N.	Fibrinogen N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.	Non-protein N.	Agglutinin titer.
	Bled.	Aborted.					I	II				
Group A.												
	days	days	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	
737	853		1.095	1.047	0.048	0.086	0.243	0.120	0.449	0.540	0.062	1:640
	955	954	1.426	1.281	0.145	0.357	0.368	0.100	0.825	0.432	0.024	1:5,120
748	797		1.123	1.085	0.038	0.194	0.256	0.132	0.582	0.441	0.062	1:640
	1,032	1,026	1.304	1.193	0.111	0.218	0.324	0.117	0.660	0.473	0.060	1:5,120
Group Bc.												
911*	888		1.035	0.968	0.066	0.077	0.222	0.115	0.414	0.504	0.051	1:1,280
	926	922	1.311	1.275	0.036	0.175	0.319	0.147	0.641	0.609	0.021	1:2,560
915	580		1.261	1.134	0.127	0.057	0.380	0.132	0.569	0.511	0.054	1:40
	831	828	1.310	1.190	0.220	0.155	0.345	0.120	0.610	0.524	0.040	1:2,560
919	826		0.958	0.930	0.028	0.055	0.231	0.098	0.384	0.484	0.062	1:1,280
	853	840	1.209	1.127	0.082	0.150	0.281	0.171	0.602	0.308	0.017	1:1,280
926	879		1.040	0.956	0.084	0.085	0.238	0.088	0.411	0.509	0.060	1:640
	886	885	1.055	0.996	0.059	0.165	0.206	0.125	0.496	0.440	0.060	1:1,280
Average before abortion.....			1.097	1.020	0.065	0.092	0.262	0.114	0.468	0.481	0.058	
Average after abortion....			1.269	1.177	0.108	0.203	0.307	0.130	0.639	0.463	0.037	

* Globulin on Cow 911 still high at 1,380 days.

It is our purpose in presenting the analyses on these cows to bring out the point, which exists also in the case of the calves presented earlier in this paper, that rather large variations may occur in the distribution of the plasma proteins of the cow, as the result of environmental factors—a term which we use for want of a better one.

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STUDIES IN YEAST METABOLISM. I.

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I.

INTRODUCTION.

The experiments described in this paper were undertaken for the purpose of investigating some of the more conspicuous changes associated with the active and rapid growth of baker's yeast (*Saccharomyces cerevisiæ*). Such a growth is essentially aerobic, and necessitates experimental conditions which fulfill this requirement, and accordingly differ from the conditions suitable for the study of alcoholic fermentation.

The methods employed permitted the construction of time-concentration curves of the most important constituents of a yeast mash which, as far as laboratory facilities would permit, represented the conditions known empirically in the yeast industry to produce the largest crops.

Lately there has been a great change in the methods of producing commercial yeast. Sugar, nitrogen, and phosphoric acid, formerly derived from grain, are now more cheaply obtained from molasses, ammonium salts, and phosphates. By very vigorous aeration of the liquid, far larger quantities of yeast are produced per unit of raw material. These "ammonia-molasses" processes have now been generally adopted by yeast manufacturers both in this country and Europe.¹

¹ A typical example of the modern ammonia-molasses process is shown in United States Patent, No. 1, 449, 127, to Nilsson and Harrison. It embodies the first technically successful method for obtaining these immense yeast crops, the production of which in the last few years has practically revolutionized the industry.

II.

General Method.

The yeast was grown on a medium composed of:

	<i>gm. per l.</i>
Beet molasses.....	48
Ammonium dihydrogen phosphate.....	1.2
“ sulfate ²	1.6

In preparation for an experiment the necessary weight of molasses to furnish 30 liters of mash was taken, diluted fourfold with water, heated to 70–75°C. with an excess of powdered chalk, filtered on a Büchner funnel, and washed. This solution, together with the salts, the yeast used for seeding the culture, and sufficient water to make 30 liters, were placed in a cylindrical stoneware crock which served as a “fermenter.” The crock was equipped with heating, cooling, and aerating coils, the last sufficient to produce a very vigorous aeration.

The molasses used in our several experiments was from the same stock supply. It contained 50 per cent sucrose, only a trace of reducing sugar, and 2 per cent nitrogen. This was present as betaines and partially hydrolyzed proteins; none was precipitable by trichloroacetic acid.

The yeast used to seed the fermenter was treated as usual with dilute sulfuric acid. As soon as the yeast was added, necessary manipulations were made as rapidly as possible, and samples of the liquid withdrawn to represent the zero hour in at most 2 minutes. The mashes were allowed to grow with constant attention from 20 to 24 hours, and similar samples withdrawn at stated intervals, usually hourly, during the run. These samples were used in determinations of yeast content, sugars, ammonia, amino and total nitrogen, alcohol, specific gravity, and total non-volatile solid matter. Sugars and ammonia were determined at once, the rest in the course of 6 or 8 hours.

The specific gravity and pH of the whole mash were also determined, and the appearance of the yeast was observed frequently under the microscope. Should the presence of foreign yeasts or bacteria become prominent, the run was naturally discarded. This happened once (in the case of Mash B).

III.

Analytical Methods.

In general, two samples were removed, one for the determination of the various soluble constituents, the other for the measurement of the yeast crop and pH. The first sample was centrifuged at once, the clear liquid removed and cooled to 2–3°.

² In the first experiment (Mash A) ammonia was used as a nitrogen source.

Total solids were determined by evaporating 10 cc. of this liquid in a shallow porcelain dish and drying to constant weight at 110°.

Reducing sugars were determined, using 10 or 25 cc. samples which, without clarification, were treated with 50 cc. of Fehling's solution according to Munson and Walker (1), the copper being finally determined as directed by Low (1). The results are expressed in grams of invert sugar per liter of mash.

Sucrose was taken as the difference between the reducing sugar and the total sugar determined in the same manner after inversion. The inversion was performed by adding 5 cc. of 38 to 39 per cent hydrochloric acid solution to 50 cc. of the liquid freed from yeast, then setting aside for 24 hours at 20–25°. The liquid was then neutralized to phenolphthalein, diluted to 100 cc., and aliquots were taken equivalent to the sample used for reducing sugars. Several determinations were tried in which the sugar was inverted in the presence of the yeast. The results were not appreciably different.

The tables of Munson and Walker are used in the computation of the sugars from the copper oxide, and the reducing sugar is computed as in the absence of sucrose. Sucrose is also expressed as grams per liter of *invert sugar*. Since the inversion is very rapid, the error appears to be small. Beet molasses contains only traces of sugars other than sucrose, and no great error has been introduced by not considering these. The probable error in the determinations of both sugars is, in our opinion, less than 1 per cent.

Alcohol was determined by distilling the mash from a small quantity of precipitated chalk until a few drops less than half of the volume had been distilled over. This was then diluted to precisely half the original sample, and the specific gravity determined with a 25 cc. gravity bottle at temperatures within half a degree of 15.6°. A correction for this deviation from the standard temperature was made by the customary formula

$$D = D' \pm d \left(0.00014 + \frac{1 - D'}{150} \right)$$

where D and D' are the apparent and the observed specific gravities, and $d = t - 15.6$, where t is the temperature of the determination in degrees Centigrade.

Ammonia and amino nitrogens were determined on the centrifuged mash liquor. For the ammonia determination 10 cc. of the solution were placed in a Folin aeration tube, made alkaline with potassium carbonate, and a current of acid-washed air was passed through and into 10 cc. of 0.1 N acid. The back titration was made with Congo red.

The total of amino and ammonia nitrogens was estimated by Sörenson's formaldehyde titration. 50 cc. of the liquid were diluted to about 150 cc., and neutralized by alkali until giving a distinct pink tinge with phenolphthalein. 50 cc. of approximately 10 per cent formaldehyde, also neutralized to phenolphthalein, were added, and the solution was brought back by standard alkali to the same end-point as before.

Nitrogen in yeast and total nitrogen of the mash were determined by the ordinary Kjeldahl method, using copper sulfate as a catalyst. For the former, dried yeast crop determinations served as samples; for the latter, 50 cc. portions of the mash, taken under precautions to prevent settling of the yeast during measurement of the sample. The determination of nitrogen in the yeast crop samples was not very satisfactory, especially at the beginning of the experiments, where the yeast crops were small. The total mash nitrogen was found to be more consistent and so replaced the former determination in our later runs.

The determination of pH on the whole mash we found could be made by adopting the collodion membrane method of Dale and Evans (2), because the mash liquid contains considerable buffer. A few cubic centimeters of the mash were dialyzed against an equal volume of distilled water for 5 to 10 minutes. Equilibrium is obtained long before much of the coloring matter has diffused through. By using brom-thymol blue or methyl red, as the case might require, the pH of the dialysate could be determined at once. Readings were made only to tenths of the pH index, and there was no attempt to prevent the escape of carbon dioxide, but the amount present at any time in the thoroughly aerated liquid was found to be very small.

Method of Determining Yeast Crops.—It seemed to us that data based upon the weight increase of the yeast would be best since the expression of our other results must necessarily be in terms of weight. This method, we are aware, has met with very little favor in the past. Pasteur, however, used it, and Bokorny (3) in recent times has used it again. Counting the cells was out of the question, since increase in the size of the individual cells is seen under the microscope to be the most potent factor in the yeast increase during the latter part of an aerated run. It is to be noted that the weight probably increases as some simple function of the cube of the cell diameter.

After many trials a procedure was adopted consisting of filtering a known volume, usually 100 cc. of mash, through a Gooch crucible, the mat of which must be composed of loose long fibred asbestos, preferably in a thick layer. Very little suction is used, otherwise the filter will clog. The cake of yeast so formed is then washed twice with 25 cc. of water and twice with 25 cc. of alcohol. The alcohol washing is necessary to remove traces of the oil originally used as a foam destroyer in the fermenter. The dead cells are dried at 110°. After drying, the material is very hygroscopic, and the crucible was placed before cooling in a tared weighing bottle of just the proper size to contain it.

Tests on the method as outlined, employing water suspensions of known amounts of yeast, gave very satisfactory results, which were practically independent of the degree of dilution of the yeast. The probable error seems to be about ± 2 per cent. Since the liquids in which our yeast was grown were well buffered,

and no very great change in pH was allowed to take place, nothing was done to regulate the acidity of the samples used for determining yeast crops. If the method were to find general use, samples before filtering would naturally have to be adjusted to nearly the same pH by appropriate buffering. The method has been carefully checked by Professor A. W. Hixson at Columbia University and found to give good results in the hands of his students.

IV.

EXPERIMENTAL.

Seven consecutive experiments of the type outlined were run. One was rejected because of infection. It was not possible to make all the determinations desired each time, and particularly after the first run, some modifications of the procedure were made. The tabulated data should make this clear. It should be noted, however, that the spent liquor (beer) from Run D was used instead of water in diluting Run F. This experiment was meant to show whether any growth-inhibiting substances had been excreted into the medium by the yeast during its growth in Run D, but no such influence was demonstrated.

On the other hand, Run E was interrupted at the 8th hour by the addition of more molasses (and ammonium salts) in order to show the effect of replenishing the food supply at the point where the original sugar had disappeared. In this way a marked increase was caused in the growth rate.

The measurement of carbon dioxide production was made on Run H, only 6 liters of medium being used in a covered vessel, the carbon dioxide being absorbed by potassium hydroxide from the issuing air current. Due to the resistance of the apparatus, only about half the usual aeration was obtained.

V.

Data.

TABLE I.

Data on Mash A.

Original composition:

Beet sugar molasses..... gm. per l. 48

 $\text{NH}_4\text{H}_2\text{PO}_4$ 1.2

Time. hrs.	pH	Reducing sugar. gm. per l.	Alcohol. gm. per l.	Yeast crop (dry substance). gm. per l.	N in yeast.* gm. per l.	Total N in beer. gm. per l.	Ammonia N in beer. gm. per l.	Specific gravity of mash.	Specific gravity of beer.	Temperature of mash. °C.	Additions during experiment.	Microscopic appearance of the yeast.
0	6.2	0	0	0.32		1.09	0.14	1.014	1.014	29.0	N added as NH_4OH .	
1	6.0	1.62		0.59		1.04	0.12	1.014	1.014	29.5	0.03	Budding well; trace of Kahm yeast.
2	5.8	4.92		0.73		1.05	0.14	1.014		30.0	0.03	Chains with no foreign organisms.
3	5.8	7.50		1.13				1.014		29.0	0.07	Clusters; no foreign organisms.
4	5.8	8.98	0	1.47	0.15			1.011	1.011	29.0	0.10	No foreign organisms.
5	5.8	5.26	1.6	2.14†	0.18			1.010		29.0	0.03	
6	5.7	4.94	6.0	3.72†				1.008	1.006	29.0		
7	5.6	1.40	7.0	4.66				1.006	1.004	29.0		
8	5.5	1.00	7.0	5.04				1.005	1.003	28.8		
9	5.6	0.53	6.0	5.27	0.43	0.93	0.17	1.006	1.004	29.0		
10	5.8	0.48		5.64				1.006	1.004	28.7		
11	5.9	0.53	5.0	5.84	0.49	0.80	0.13	1.006	1.004	28.8		
12	6.1			6.02				1.006	1.004	28.7	H_2SO_4	

13	6.3	0.51	5.0	6.09	0.51	0.80	0.12	1.007	1.005	28.8	H ₂ SO ₄	Pairs; no foreign organisms.
14	6.4			6.23	0.47			1.007	1.004	28.7		Fewer buds; no foreign organisms.
15	6.3			6.28		0.69	0.10	1.007	1.004	28.6		Very few buds; trace of Kahl.
16	6.3	0.48		6.68				1.007	1.005	28.5		No foreign organisms.
17	6.2		1.0	6.76	0.55	0.69	0.07	1.008	1.004	28.7		" "
18	6.2			6.97				1.008	1.005	29.0		" "
19	6.2			7.38		0.70	0.06	1.007	1.005	29.0		Cells discrete; no foreign organisms.
20	6.1	0.52		7.44				1.008	1.005	28.5		No foreign organisms.
21	6.2			7.61	0.58	0.69	0.07	1.007	1.005	28.5		" "
22	6.1			8.32	0.54			1.006	1.005	28.5		" "
23	6.4			8.12		0.68	0.04	1.007	1.005	28.7	H ₂ SO ₄	" "
24	6.2			7.90		0.65	0.05	1.007	1.005	28.6		" "
25	5.6	0.44		8.14	0.58	0.63	0.05	1.007	1.004	28.6		" "

* Expressed as grams of N in the yeast per liter of mash.

† Respectively 4½ and 6.17 hours.

TABLE II.
Data on Mash C.

Original composition:

Molasses.....	gm. per l.	48
NH ₄ H ₂ PO ₄	1.2	
(NH ₄) ₂ SO ₄	1.6	

Time.	pH	Reducing sugar.	Total sugar.	Alcohol.	Total solids.	Yeast crop (dry substance).	N in yeast.	Total N in beer.	Formol N in beer.	Ammonia N in beer.	Amino N in beer.	Specific gravity of mash.	Specific gravity of beer.	Temperature of mash.	Additions during experiment.	Microscopic appearance of the yeast.
hrs.		gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.			°C.		
0	5.8	25.39	0	41.59	0.55		1.51	0.55	0.47	0.09	1.017	1.017	1.017	29	NaHCO ₃	Trace of Kahm in stock.
2	5.9	4.05	22.59		0.79		1.40	0.53	0.46	0.07	1.015	1.015	1.015	29		" "
4	5.2	9.99	20.82	1.63	4.78	1.51	1.14	0.38	0.50	0.47	0.03	1.013	1.013	28		" "
6	5.0	8.83	14.56	4.0		3.19	0.31	1.20	0.41	0.37	0.04	1.011	1.010	27	1.5	Apparently pure culture; no Kahm.
8	5.6	0.73	1.14	6.5	27.43	5.53	0.34	1.00	0.26	0.23	0.03	1.008	1.006	28		" "
10	5.8	0.71	0.58	5.5		6.35	0.53	0.86	0.19	0.15	0.04	1.008	1.006	27		" "
12	6.0	0.43	0.51	5.5	15.32	6.65	0.57	0.85	0.16	0.11	0.04	1.008	1.006	28		Few small cocci; otherwise good.
14	6.5	0.42	0.52	3.0		7.21	0.72	0.79	0.13	0.08	0.05	1.006	1.005	27		No change.
16	6.0	0.43	0.56		14.61	7.42	0.72	0.71	0.12	0.08	0.04	1.007	1.005	28		" "
18	6.2	0.43	0.54			7.89	0.65	0.75	0.10	0.09	0.01	1.008	1.005	29		" "
20	6.3	0.43	0.54		14.76	8.04	0.70	0.72	0.08	0.06	0.02	1.008	1.006	29		" "

TABLE III.

Data on *Mash D*.

Original composition:

Molasses.....	gm. per l.
$\text{NH}_4\text{H}_2\text{PO}_4$	48
$(\text{NH}_4)_2\text{SO}_4$	1.2
	1.6

Time.	pH	Reducing sugar.	Total sugar.	Alcohol.	Total solids.	Yeast crop (dry substance).	N in yeast.	Total N in beer.	Formol N in beer.	Ammonia N in beer.	Amino N in beer.	Specific gravity of mash.	Specific gravity of beer.	Temperature of mash.	Additions during experiment.	Microscopic appearance of the yeast.
hrs.		gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.			°C.		
0	6.0	0.13	24.17		39.85	0.47	1.45	0.55	0.48	0.07	1.014	1.014	1.014	28	NaHCO ₃	Apparently pure culture.
2	5.7	6.82	22.77		36.57	0.75		0.55	0.49	0.06	1.014	1.014	1.014	29		
4	5.3	12.69	18.74	3.0	31.74	1.55	0.13	1.27	0.49	0.43	0.06	1.013	1.012	28		
5		10.95	14.11					0.45	0.29					28	1.0	"
6	5.0	8.93	8.21	8.0		3.46	0.28	1.12	0.37		0.08	1.011	1.008	28		
7		3.00*	3.36*													
8	4.2	0.80	0.89	10.0	15.50	4.92	0.42	0.98	0.31	0.26	0.05	1.008	1.005	27	0.5 H ₂ SO ₄	"
9	5.0			6.5		5.30	0.47	0.77	0.25	0.17	0.08	1.006	1.005	29		
10	5.0	0.28	0.54													
12	6.4	0.42	0.55	6.5	15.54	5.58	0.48	0.85	0.21	0.15	0.06	1.006	1.004	30	0.2	"
13	6.6															
14	6.0			4.0		5.97	0.51	0.73	0.20	0.13	0.07	1.005	1.004	29		
16	6.2	0.44	0.56		14.02	6.14	0.52	0.78	0.20	0.16	0.04	1.006	1.005	30	"	"
18	6.2	0.33	0.58			6.45	0.51	0.78	0.18	0.12	0.06	1.008		28		
20	6.4	0.43	0.50		14.05	6.76	0.53	0.78	0.18	0.11	0.07	1.008	1.006	29		
22	6.4		0.51			6.80	0.54	0.75	0.17	0.11	0.06	1.007	1.007	29	"	"
24	6.2	0.37	0.48		13.43	7.46	0.54	0.68	0.14	0.09	0.05			30		

* At 6½ hours.

TABLE IV.
Data on Mash E.

Original composition:

Molasses.....	gm. per l.
$\text{NH}_4\text{H}_2\text{PO}_4$	48
$(\text{NH}_4)_2\text{SO}_4$	1.2
	1.6

Time.	pH	Reducing sugar.	Total sugar.	Total solids.	Yeast crop (dry substance).	Total N in beer.	Formol N in beer.	Ammonia N in beer.	Amino N in beer.	Total N of mash.	Specific gravity of mash.	Temperature of mash.	Additions during experiment.	Microscopic appearance of the yeast.
hrs.		gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.		°C.		
0	5.6	0.96	24.30	42.20	0.54	1.39	0.55	0.44	0.11	1.41	1.014	27	NaHCO ₃ 0.5 2.0	Stock looks pure. Little change. Many buds; no infection.
2	6.0	9.66	22.30		0.94		0.51	0.41	0.10		1.014	29		
4	5.6	12.09	16.70	32.33	2.06		0.46	0.38	0.08		1.014	26		
6	4.8	4.92	6.13		4.08		0.34	0.26	0.07		1.008	30		
8	4.8	0.72	2.30	15.80	5.15	0.93	0.24	0.21	0.04	1.37	1.008	29		

Additions calculated to new volume made 8 to 8½ hrs.

	cc.	gm. per l.
Old volume.....	4,790	Molasses..... 36
New "	5,360	NH ₄ H ₂ PO ₄ 0.65
		(NH ₄) ₂ SO ₄ 0.89

8½	5.6	4.32*	17.33	40.40	1.63	0.53	0.40	0.13	2.02	1.018	28	NaHCO ₃ 1.0	Yeast looked healthy and grew well, but contained a trace of Kahm throughout.
9													
10½		0.89	1.25			0.35							
11	6.8			7.32							28		
12	6.5										27		
12½	6.8	0.74	0.88	26.41	7.54	0.37	0.19	0.18		1.012	29		
14½	6.8			7.90							29		
16	6.5	0.67	0.76	24.92	8.00	1.36	0.30	0.22	0.08	1.98	30		

* Concentrations from 8½ hrs. on are those observed in the liquids analyzed. No corrections are made here on account of the change in volume.

TABLE V.
Data on Mash F.

Original composition:

Molasses.....	gm. per l.
	48
NH ₄ H ₂ PO ₄	1.2
(NH ₄) ₂ SO ₄	1.6

This mash differs from other mashes in that the beer from Mash D was used instead of water.

Time.	pH	Reducing sugar.	Total sugar.	Total solids.	Yeast crop (dry substance).	Total N in beer.	Formol N in beer.	Ammonia N in beer.	Specific gravity of mash.	Temperature of mash.	Additions during experiment.	Microscopic appearance of the yeast.
hrs.		gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.	gm. per l.		°C.		
0	5.8	3.60	22.99	50.41	0.54	1.85	0.65	0.61	1.019	31	NaHCO ₃	Yeast healthy and
2	5.6	15.22	21.64				0.63	0.56	1.019	29	1.0	apparently growing
4	5.2	9.82	10.32	37.62	3.17		0.61	0.49	1.016		1.0	well. A few Kahl
6	5.2	3.65	3.58		3.25*		0.48	0.43	1.014	27	1.0	and bacterial cells
					3.87*							present throughout.
8	6.0	3.80		25.03	4.59	1.34	0.42	0.30	1.013	29		

* At 5½ and 6½ hours respectively.

TABLE VI.
Data on Mash H.

Original composition:

Molasses	<i>gm. per</i>
$\text{NH}_4\text{H}_2\text{PO}_4$	48
$(\text{NH}_4)_2\text{SO}_4$	1.2
	1.6

Time.	pH	Reducing sugar.	Total sugar.	Total solids.	Yeast crop (dry substance).	Temperature of mash.	CO_2 evolved calculated to original volume.	Microscopic appearance of the yeast.
		<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	$^{\circ}\text{C}.$	<i>gm. per l.</i>	
hrs.								
0		0.24	23.56	39.40	0.36	29	0	Healthy and apparently pure culture.
1	6.0	3.78	22.88	37.87			0.07	
2	6.0	10.80	21.58	36.80	0.54	29	0.20	
3	5.6	15.01	20.14	35.02		28	0.51	
4	5.2	15.29	18.13	31.91	1.21	28	0.91	"
5	5.0	13.14	14.08	27.13		29	1.72	"
6	5.4	7.53	8.37	20.91	2.58	29	3.09	
7	5.6	2.56	2.34	16.26		29	4.48	
8	6.0	0.33	0.42	15.21	3.78	29	5.04	"
9						29	5.08	
10	5.8	0.55	0.26	15.19	4.23	29		
11						28	5.12	
12	6.3			14.75	3.81	28		
13						28	5.20	"
14	6.3			13.86	3.91	28		"
15						29	5.24	
16	6.1	0.54	0.05	13.69	3.85	29	5.31*	"

* Including residual CO_2 in mash.

VI.

Rate of Yeast Growth.

The existence of a logarithmic phase of growth as pointed out by Slator and others (4) is clearly indicated. After a preliminary lag phase, lasting about an hour, the growth is logarithmic up to about the 8th hour, when an abrupt change in the rate takes place. After this the yeast continues to grow; that is, to increase in weight, at a greatly reduced velocity which, however, is also fairly constant. By plotting the logs of the yeast crop against the time, the occurrence of logarithmic growth

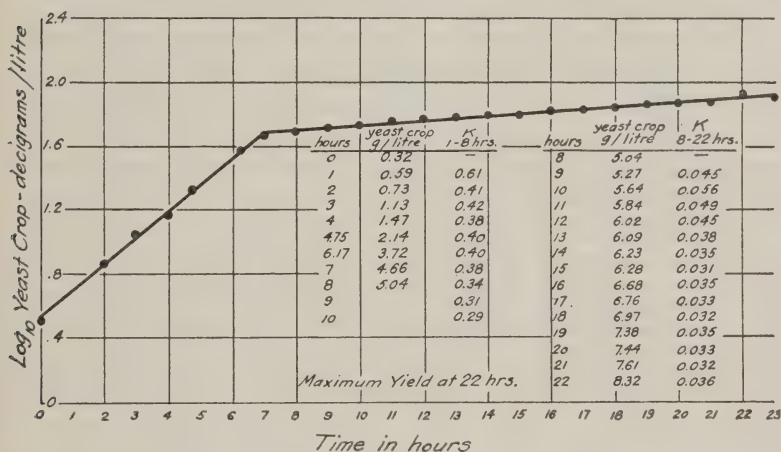


FIG. 1. Mash A. Yeast crop curves.

will be shown by a straight line (Fig. 1). Instead of depicting these data graphically, the formula $Kt = \log(a + x) - \log a$ may be used to calculate values of K in terms of the time t , and the initial (a) and final ($a + x$) yeast concentrations. K is seen to be a constant for the first 8 hours, and also a constant from the 8th or 9th hour until apparent growth practically stops.

The values of the growth rate constant, when calculated from weight data, show the same division into a period of rapid growth and a period of slow growth, that Slator observed from cell count data; but the weight increase follows the logarithmic law throughout the period of slow growth as well. That is to say, the increase

in weight of the yeast is at no time irregular or sporadic, but despite the changes occurring in the concentrations of foodstuffs present, proceeds at a constant rate throughout the latter two-thirds of the fermentation. Under conditions of our experiments the growth rate during the slow period was found always to be approximately one-tenth the value during the rapid period.

There is evidently a transition period occurring about the 8th hour of growth, which is marked by a change from rapid to slow increase in weight. This transition is not always abrupt, but may last somewhat over an hour (Mash C). Consequently, a more accurate picture is perhaps obtained by calculating the growth constant from the 10th hour (Tables VII and VIII).

This transition is coincident with the disappearance of sugar³ with the maximum concentration of alcohol, and the attainment of the minimum specific gravity and minimum amount of total solid matter (Figs. 2 and 3). The connection between the sugar and total solid matter is obvious, as is that between minimum solid matter, maximum alcohol, and minimum specific gravity.

The presence of considerable alcohol may in itself have a slowing effect on the growth velocity, as has often been stated. It cannot, however, be the only factor, for the alcohol concentration subsequently decreases, without apparent increase in growth rate. The accumulation of other waste products would also tend to slow down the multiplication, but this is not in itself a satisfactory explanation. In Mash F, Table V, yeast was successfully grown upon the filtered beer of a previous experiment (Mash D, Table III) with an initial growth velocity of 0.42.

The material composing Mash F should have contained all the non-volatile substances deleterious to growth produced in running Mash D for 20 hours. As a matter of fact, the yeast grew *better* on this medium than on the regular medium used (Table IX). The difficulty of keeping such a medium sterile, however, is considerable, and the experiment was not continued long because the presence of bacterial infection was beginning to show. With a strain of yeast capable of resisting the encroachment of such infection, the presence of the spent liquor is apparently an ad-

³ The small quantity of sugar remaining is not metabolized rapidly. This is discussed in more detail later.

TABLE VII.

Values of Growth Constant Calculated for the First 8 Hours (AB, Fig. 2), and Then Separately for the Final 12 Hours (BC, Fig. 2) (Mash C).

Time.	<i>K</i> (calculated from 0 hr.).	
<i>hrs.</i>		
0		
2	0.18	
4	0.25	
6	0.29	
8	0.29	
	<i>K</i> (calculated from 8th hr.).	<i>K</i> (calculated from 10th hr.).
8		
10	0.069	
12	0.046	0.023
14	0.044	0.031
16	0.037	0.026
18	0.036	0.027
20	0.031	0.024

TABLE VIII.

Values of the Growth Constant Calculated Separately for Each Portion of the Growth Curve (Mash D).

Time.	Yeast crop.	Growth constant.	$K = \frac{\log(a+x) - \log a}{0.434 t}$
<i>hrs.</i>	<i>gm. per l.</i>		
0	0.47		Calculated from 0-8 hrs. Curve AB (Fig. 3).
2	0.75	0.24	
4	1.55	0.30	
6	3.46	0.33	
8	4.92	0.30	
8	4.92		Calculated from 8-24 hrs. Curve BC (Fig. 3).
10	5.30	0.037	
12	5.58	0.031	
14	5.97	0.032	
16	6.14	0.028	
18	6.45	0.028	
20	6.76	0.026	
22	6.80	0.023	
24	7.46	0.026	

vantage. There seems little basis for assuming the presence of toxic substances in the used mash liquor of our experiments.

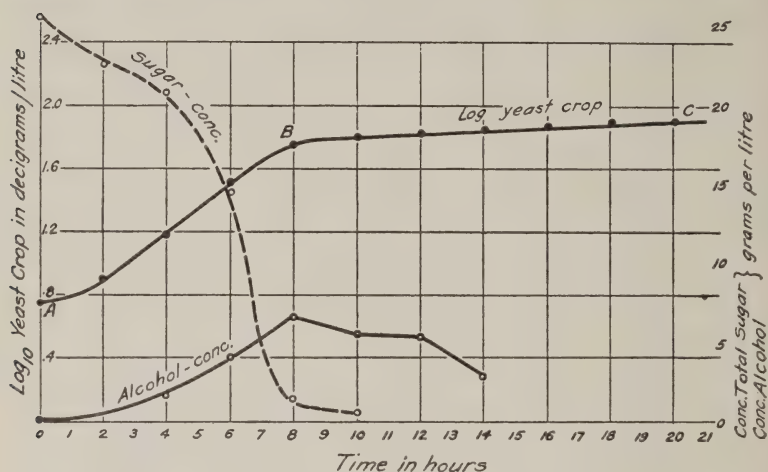


FIG. 2. Mash C. Yeast yield curves.

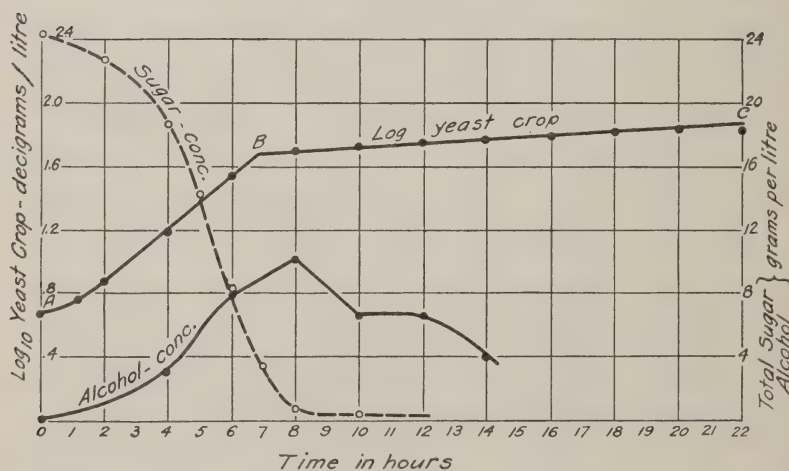


FIG. 3. Mash D. Yeast crop and total sugar curves.

Curves of the sugar concentration in Experiments C and D show that the period of rapid growth is the period during which

there is an appreciable quantity of sugar present in the solution. This correspondence seems to indicate an important factor in yeast growth. In spite of the fact that our data show the ability of yeast to grow on practically sugar-free media, it is evident that the rate of this growth and the corresponding "fixation"

TABLE IX.

Effect of Previously Used Culture Medium on Rate of Yeast Growth (Mash F Composed of Yeast and Filtered Beer from Mash D, Together with More Molasses and Inorganic Salts).

Time.	Yeast crop.	K
hrs.	gm. per l.	
0	0.54	
2	1.26	0.42
4	3.17	0.43
6	3.55	0.31
8	4.59	0.25

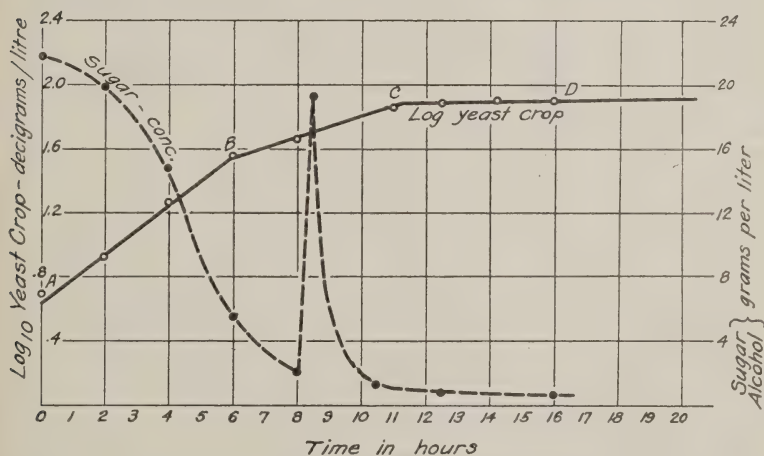


FIG. 4. Mash E. Yeast yield curve.

of nitrogen—by the way—is accelerated roughly to ten times its value by the presence of invert sugar.

An experiment was made, therefore, in which additional sugar was added at the 8th hour, and the growth rate observed subsequent to this addition (Table X). Accordingly, in Mash E more

molasses was added between the hours 8 and $8\frac{1}{2}$, and the curve of Fig. 4 was obtained.

The rapidity of sugar removal was surprising, and made accurate observations difficult. The growth rate constant was, however, definitely held up, though not as high as in the first stage of fermentation. It drops during the presence of the additional sugar to about one-third the former value, and after utilizing this sugar, to the usual value of about one-tenth. The yeast crop determinations were made at rather ill-chosen times, and there may be only two parts to the curve where we have indicated three. In any case, the accelerating effect of the sugar seems clearly shown.

TABLE X.

Showing the Effect of Replenishing the Sugar Supply on Yeast Growth (Mash E).

Time.	Yeast crop (corrected to final volumes).	K		
		Curve AB.* 0-6 hrs.	Curve BC.* 6-11 hrs.	Curve CD.* 11-16 hrs.
<i>hrs.</i>	<i>gm. per l.</i>			
0	0.48			
2	0.84	0.28		
4	1.84	0.34		
6	3.65	0.34		
8	4.60	0.28	0.12	
11	7.32		0.14	
$12\frac{1}{2}$	7.54			0.020
$14\frac{1}{4}$	7.90			0.024
16	8.00			0.018

* See Fig. 4.

VII.

Observations on the Sugar Metabolism.

No attempt was made to trace the sugar beyond its disappearance, and consequently no addition has been made to the subject of intermediate products of alcoholic fermentation.

There is an unfermentable reducing substance produced during the course of the yeast growth. Before the yeast is added to the diluted molasses and salts, only traces of substances capable of reducing Fehling's solution are found (Figs. 5 and 6). As soon as the yeast is introduced inversion of the cane-sugar commences and

the solution possesses a definite and at times very large capacity for reducing cupric salts. As the growth progresses and the invert sugar is decomposed, the copper-reducing capacity of the liquid decreases, until finally a minimum is reached. This usually amounted to half a gram per liter (calculated as invert sugar), much larger than the reducing power of the liquid either just

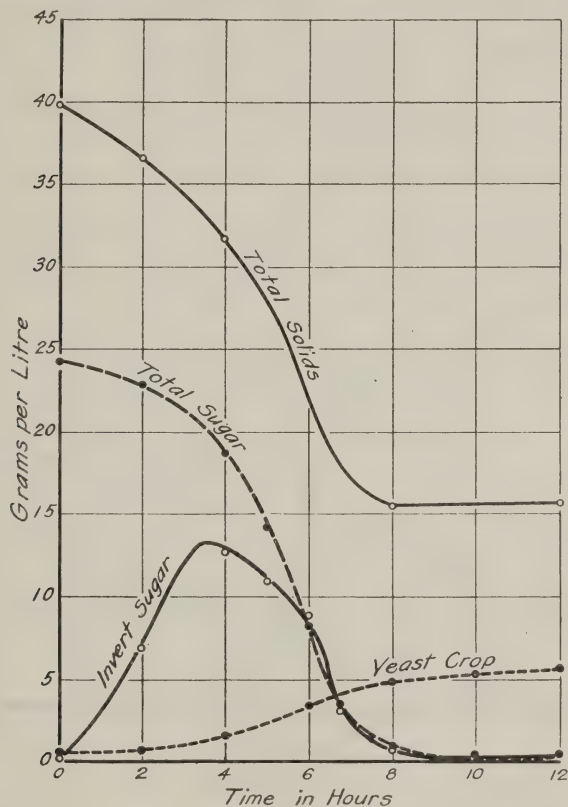


FIG. 5. Mash D.

prior to or just after the first introduction of yeast. The material is, therefore, a result of the yeast growth. Similar substances have been referred to as "unfermentable reducing sugar" in the molasses, but strictly speaking, this cannot be. As far as our experiments go the substance may not necessarily be a sugar at all. The amount remains practically constant during the last two-thirds of the experiment; that is to say, for twice the length

of time required to ferment out the sugar originally present. This gives reason for supposing that it is not assimilable or acted upon by the yeast, and appears to bar the possibility of aldehydes and similar intermediate products of alcoholic fermentation.

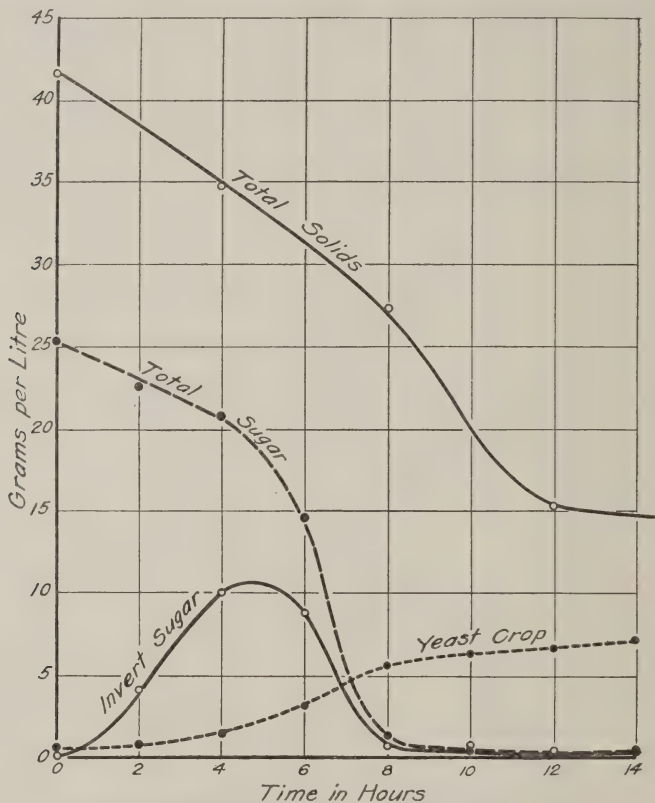


FIG. 6. Mash C.

The sugar originally present in our experiments lasted uniformly for about 8 hours. It has been pointed out that the point of sugar disappearance seems to represent a real critical point in the growth of the organism. Subsequently, the yeast is able to grow and, as can be seen from microscopic observations, to multiply as well. The yeast, therefore, is capable of utilizing as a source of carbohydrate the products formed by breaking down the sugar, although on such a medium, the growth is not as

rapid. These intermediate products are volatile from the faintly acid liquid of the mash at 110° . The total (non-volatile) solid matter of the mash liquor, obtained by separating the yeast and drying the material to constant weight at 110° , shows this. In the accompanying curves (Figs. 5 and 6), the drop in non-volatile solids corresponds to that in the total sugars. The amount of nitrogenous matter removed is so small compared with the loss due to sugar that the curve presented is hardly accurate enough to more than indicate it. The sugar is evidently eventually replaced by volatile material, although there may be indications of the *temporary* presence of non-volatile substances derived from it shown in Mash C, where the loss in sugar is slightly more rapid than in total solids.

Sugar Concentration and Yeast Growth.—Working with non-aerated mashes of grain worts, Slator (5) has shown that the yeast growth and the sugar decomposition are related, assuming that the individual cell has a constant fermenting⁴ (*i.e.* sugar-destroying) activity. Since the logarithmic law of yeast growth has been found valid when measuring the weight of yeast produced, this assumption may be made correspondingly for a unit weight of yeast. The period through which the measurements are made (that of the presence of sugar in the liquid) is also that of the rapid logarithmic phase. The development of the mathematical expression is the same as far as we need to carry it.

In a medium containing at the start of an experiment a grams of yeast, the amount of yeast present at any succeeding time, t , can be denoted $a + x$. The relation between these two quantities is $Kt = \log_e \frac{(a + x)}{a}$, when K is the growth constant of the yeast.

If F represents the grams of sugar fermented in unit time by unit weight of yeast, and S the total sugar fermented in the time t , then

$$S = \int_0^t (a + x) F dt$$

$$\text{or } F = \frac{KS}{x}$$

⁴ We have retained Slator's nomenclature in this connection but since our criterion of "fermentation" here is simply the disappearance of sugar, we cannot distinguish between the sugar actually fermented and that assimilated by the yeast. The CO_2 evolution as a criterion of fermentation in an aerated mash is, we have found, also open to objection.

From yeast crop measurements we observe x and can calculate K ; from sugar determinations, S can be found. Since F is also a constant, we may combine these two and write $S = Cx$ where C is a third constant. A constant such as C will, however, vary with variations in K , but in our experiments while sugar remained in the medium K did not vary materially. This case is the opposite of that obtaining for the nitrogen concentrations, as we shall later see.

The fermenting capacity (F in the Tables XI and XII) is shown to be very nearly constant, although a small but definite increase is seen, reaching its maximum at the period of maximum invert sugar concentration. In a yeast unable to ferment sucrose, this would be expected and may serve as added evidence of the inability of *cerevisiae* to do this. The fermenting capacity of the cells might not be utilized fully by a solution containing an inadequate supply of invert sugar, as is the case in the beginning of the experiments.

On the contrary, the values for C are always abnormally high at the start, due to the initial lag in the growth rate.

No account is taken of whether the sugar serving the yeast as a source of carbon is assimilated directly by the yeast or not. According to Bokorny (3) this may occur with 1 per cent of the sugar present, under conditions similar to those employed in this work. It is probable that the greater part of the sugar is fermented, and judging by the small quantities of alcohol obtained compared with the sugar used, the yeast finds its food in the intermediate substances between sugar and alcohol, and perhaps even from the last named substance in considerable amount.

Inversion of Sucrose.

While there appears to be a definite connection between concentration of yeast and speed of fermentation, there is no similar connection evident in regard to the speed of inversion.

We have computed the amount of sugar inverted on the assumption that no sucrose was used directly by the yeast cells. With this assumption the difference between the invert sugar and the total sugar determined at any time will show the sucrose remaining in solution; and if this is deducted from that originally present (*i.e.* at zero hour) the difference will represent the sucrose inverted.

TABLE XI.

Constancy of the Sugar-Fermenting Capacity of Growing Yeast.

Time.	Increase in yeast weight.	Sugar used.	Growth rate constant.	Fermenting capacity of yeast.	Growth and fermentation constant.
t	x	S	K	$F = \frac{SK}{x}$	$C = \frac{S}{x}$

Mash C.

hrs.					
0	0	0			
2	0.24	2.80	0.18	2.18	11.6
4	0.96	4.57	0.25	1.21	4.78
6	2.64	10.83	0.29	1.20	4.10
8	4.98	24.25	0.29	1.40	4.86
10	5.80	24.81	0.25	1.05	4.28

Mash D.

0	0	0			
2	0.28	1.40	0.24	1.18	5.00
4	1.08	5.43	0.30	1.48	5.03
5	1.83*	10.06	0.32	1.74	5.46
6	2.99	15.96	0.33	1.77	5.32
6 $\frac{3}{4}$	4.11	20.81	0.34	1.71	5.07
8	4.45	23.28	0.30	1.54	5.24
10	4.83	23.63			

Mash E (two additions of sugar).†

0	0	0			
2	0.36	1.85	0.28	1.44	5.14
4	1.36	6.85	0.34	1.69	5.04
6	3.17	16.27	0.34	1.74	5.13
8	4.12	19.7	0.28	1.35	4.78
8 $\frac{1}{2}$	4.65*	22.4	0.28	1.35	4.83
10 $\frac{1}{2}$	6.44*	38.0	0.25	1.49	5.90
12 $\frac{1}{2}$	7.06	39.0	0.22	1.22	5.53

Mash H.

0	0	0			
1	0.04*	0.68	0.11	1.80	17.0
2	0.18	1.98	0.20	2.24	11.0
3	0.44*	3.42	0.27	2.08	7.8
4	0.85	5.43	0.30	1.95	6.4
5	1.42*	9.48	0.31	2.04	6.7
6	2.22	15.19	0.33	2.26	6.8
7	3.44*	21.22	0.34	2.09	6.2
8	3.42	23.14	0.30	2.00	6.8
10	3.87	23.30	0.25	1.48	6.0

* Value interpolated from yeast crop curve.

† All quantities calculated to the final volume to avoid discrepancy due to dilution made at 8 $\frac{1}{2}$ hours.

If we were to postulate that a given weight of yeast possessed a constant inverting capacity instead of a fermenting capacity, as we have done, we would again have an expression $F' = \frac{S'K}{x}$ where F' would signify an inverting capacity of the yeast, and S' the amount of sucrose inverted. We could also write in a similar manner $C' = \frac{S'}{x}$ to denote a constant dependent on both F' and S' .

As an example, the calculations are given for one experiment only, but the type of result is no different in the other cases (Table XII).

The fairly constant values for F indicate that fermentation is a function of the yeast cell, while the varying values of F'

TABLE XII.
Variation of the Inverting Capacity of Growing Yeast (Mash D).

Time.	Yeast crop.	Increase in yeast.	Sugar inverted.	Calculated for fermentation.		Calculated for inversion.	
t	$a+x$	x	S'	$F = \frac{SK}{x}$	$C = \frac{S}{x}$	$F' = \frac{S'K}{x}$	$C' = \frac{S'}{x}$
<i>hrs.</i>							
0	0.47	0					
2	0.75	0.28	8.09	1.18	5.00	6.82	29.0
4	1.55	1.08	17.99	1.48	5.03	4.90	16.7
5	2.30*	1.83	20.88	1.74	5.46	3.62	11.4
6	3.46	2.99	24.04	1.77	5.32	2.68	8.1
6 $\frac{3}{4}$	4.58*	4.11		1.71	5.07		

* Value interpolated from yeast crop-time curve.

and C' imply that invertase action is not a function of the yeast cell; in other words, that the difference in the behavior of the two "constants", F and F' , points to the intracellular nature of the zymase, and to the extracellular nature of the invertase. The contention first made by O'Sullivan (6) that invertase action in the case of healthy and unbroken yeast cells occurs at the site of the cells, does not seem to be corroborated.

The rate of sugar inversion itself is surrounded in these experiments with so many other changing factors that its consideration is difficult. The quantity of invertase in the solution is obviously increasing with the quantity of yeast. The con-

centration of saccharose is decreasing, and the invert sugar is being removed almost as fast as it is formed.

One would expect the velocity of the reaction to increase with decreasing concentrations of sucrose, provided the quantity of invertase is small; this at first must be the case. The velocity of the reaction will also increase with increasing quantities of yeast, but when the amount of invertase becomes large, the first factor will cease to be important, so that the acceleration of the reaction due to increasing concentration of yeast will not be proportional to the growth of the yeast, but rather less (7). The reaction velocity will tend to increase, but the actual weight of sugar inverted will be lessened because of the diminishing concentration of sucrose.

TABLE XIII.
Velocity of Inversion (Mash C).

Time.	Velocity constant.		<i>S</i>	<i>S'</i>	Observed rate of inversion per hr.
	Calculated from $K = \frac{\log S - \log (S - S')}{0.434 t}$	Calculated from $K = \frac{\log S - \log (S - S')}{0.434 x}$			<i>V</i>
					<i>gm. per l.</i>
<i>hrs.</i>					
0			25.40		
2	0.157	1.13		6.85	3.43
4	0.228	0.95		14.6	3.65
6	0.250	0.57		19.7	3.28
8	0.520	0.83		25.0	3.12*

* These values for *V* are deceptively close in agreement, however. In other experiments *V* varies by as much as 50 per cent.

We have observed an increase in the velocity constant of the inversion, calculated from the ordinary formula, $0.434 Kt = \log S - \log (S - S')$, where *S* is the original sucrose concentration, and *S* - *S'*, the concentration at time, *t*. This increase, shown in Table XIII, is not proportional to the increase in the yeast, but less; for if the assumption be made that $\frac{dS'}{dt} = K (a+x) (S - S')$ (the velocity of inversion proportional to the concentration of the sucrose and also to the amount of yeast present), then on integration $K' = \frac{\log S - \log (S - S')}{0.434 x}$, and values for *K'* decrease as the yeast increase *x* grows greater.

It happens, probably by chance, that in one case these two tendencies approximately counterbalanced each other, so that the actual rate of inversion observed is almost constant. See V in the Table XIII (grams of sucrose per liter inverted per hour).

VIII.

Observations on Nitrogen Metabolism.

Assertions that baker's yeast grown in a current of air is able to fix atmospheric nitrogen have been made many times, and as frequently contradicted. Unless there is such a fixation, the total

TABLE XIV.

Progressive Utilization of Soluble Nitrogen by the Growing Yeast (Mash A) and Constancy of the Total Nitrogen Present.

Time.	N in yeast.	N in filtrate.	Total N in yeast + filtrate.	Total N of mash by independent determination.
<i>hrs.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>
0		1.45		
4	0.13	1.27	1.40	
6	0.28	1.12	1.40	
8	0.42	0.98	1.40	
10	0.47	0.77	1.24	1.25
12	0.48	0.85	1.33	1.39
14	0.51	0.73	1.24	1.29
16	0.52	0.78	1.30	1.36
18	0.51	0.78	1.29	1.36
20	0.53	0.78	1.31	1.46
22	0.54	0.75	1.29	1.29
24	0.54	0.68	1.22	1.29

nitrogen of the mash liquor should decrease by the amount stored in the yeast cells, but the total nitrogen of the liquid plus that of the yeast should remain constant. Independent determinations of the total nitrogen found in our mashes with that found in the yeast and in the filtrate from the yeast are shown in Table XIV.

In the case of Mash A, where ammonia was added at intervals during the first 7 hours, there is an evident loss of nitrogen at first, due, we think, to volatilization in the air current.

In the later period of Mash A and throughout experiments using ammonium sulfate, the total nitrogen was constant. This indicates to us that claims of the ability of *cerevisiæ* to fix at-

mospheric nitrogen are without foundation. Similar figures for the total nitrogen of another experiment are given in Table XV.

Let us now consider the changes which occur in the partition of this nitrogen. The nitrogen content of the yeast is reasonably constant, and the amount of nitrogen fixed by the yeast in in-

TABLE XV.
Total Nitrogen of the Mash Remains Constant (Mash E).

Time.	Total N of mash by direct determination.
hrs.	gm. per l.
0	1.41
8	1.37
Additional molasses and salts added.	
8½	2.02
16	1.98

TABLE XVI.
Comparing Decrease in Soluble Nitrogen with Yeast Increase (Mash C).

Time.	Yeast increase.	Decrease in total soluble N in mash.	Decrease in soluble N Yeast increase	Decrease in soluble N Yeast increase $\times K$.
hrs.	gm. per l.	gm. per l.		
0				
2	0.24	0.108	0.45	0.081
4	0.96	0.126	0.131	0.033
6	2.64	0.302	0.114	0.033
8	4.98	0.506	0.101	0.029
10	5.80	0.646	0.111	0.027
12	6.10	0.654	0.107	0.022
14	6.66	0.712	0.107	0.019
16	6.87	0.792	0.115	0.019
18	7.34	0.759	0.103	0.015
20	7.49	0.786	0.105	0.014

soluble form will depend upon the quantity of yeast grown. In other words, $\frac{\text{decrease in soluble N}}{\text{yeast increase}} = \text{a constant.}$

This resembles in form the ratio $\frac{S}{x}, \frac{(\text{sugar used})}{(\text{yeast increase})}$, which has just been discussed. In the case of the sugar, the ratio is

constant when K for the growth rate remains constant. The ratio $\frac{\text{decrease in soluble N}}{\text{yeast increase}}$ is a constant, however, throughout the entire experiment, so the utilization of the nitrogen is independent of the growth rate of the yeast. This conclusion is

TABLE XVII.
Comparing Decrease in Soluble Nitrogen with Yeast Increase
(Mashes A, D, E, and F).

Time. <i>hrs.</i>	Ratio.			
	Mash A.	Mash D.	Mash E.	Mash F.
1				
2		0.250		
3				
4		0.166		
5				
6		0.110		
7				
8		0.105	0.100	0.126
9				
10		0.140		
11	0.227			
12		0.117		
13	0.149			
14		0.131		
15	0.236			
16		0.118	0.094	
17	0.154			
18		0.112		
19	0.107			
20		0.106		
21	0.101			
22		0.110		
23	0.108			
24	0.104	0.110		
25	0.104			

perhaps to be expected (8), but its demonstration shows that the utilization of the soluble nitrogen represents nothing more than building up of yeast tissue. There is no storage, strictly speaking, in the cells, nor does the yeast in the period of its rapid growth in the presence of sugar require more or less nitrogen than during

the restricted growth of the older mash. There is no apparent protein-sparing action of the carbohydrate. The constancy of the ratio is shown in Tables XVI and XVII, and applies throughout each entire experiment, since unlike the sugar case, an excess of nitrogen continues to the end. An expression containing the growth K , similar to $\frac{KS}{x}$ would lead to varying values, as can be readily seen by inspection. An example is shown in the last column, Table XVI.

The growing yeast abstracts from the solution amounts of nitrogen depending solely on the quantity of yeast grown, not on the speed of that growth. From what nitrogen source is this

TABLE XVIII.

Decrease in Formaldehyde-Titratable Nitrogen and in Total Soluble Nitrogen, and the Ratio between Them (Mash C).

Time.	Decrease in formol N.	Decrease in total soluble N.	$\frac{\text{Decrease in formol N.}}{\text{Decrease in total N.}}$
<i>hrs.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	
0			
2	0.027	0.108	0.25
4	0.056	0.126	0.44
6	0.147	0.302	0.49
8	0.296	0.506	0.59
10	0.360	0.646	0.56
12	0.396	0.654	0.60
14	0.424	0.722	0.59
16	0.436	0.792	0.55
18	0.456	0.759	0.60
20	0.472	0.786	0.60

by preference derived? We can divide the soluble nitrogen of the mash liquor into two parts: first, the "formol nitrogen," which consists of NH_2 groups easily attacked by formaldehyde; and the "non-formol nitrogen," constituting the remainder. In the medium there is at the start a reasonable excess of both. Fulmer, Nelson, and Sherwood (9) have shown that the ammonium salts represent all the nitrogen essential to a meager reproduction. The formol nitrogen certainly would seem the more easily assimilable, and presumably the form of food which would of choice be drawn upon first by the yeast. Tables XVIII and XIX show

that this is not altogether the case. Here the decrease in formol N and the decrease in total soluble N are shown parallel, together with the ratio between them. It is curious that this ratio appears to be approximately constant throughout the series of experiments. Even at the outset, the yeast, in the presence of a perfectly adequate supply of amino groups which it can use, chooses a fairly definite proportion of the presumably more "indigestible" non-formol nitrogen.

If there were present a certain portion of the beet molasses nitrogen which is available for yeast growth, this should show signs of final exhaustion, particularly since it is known that any

TABLE XIX.
Value of Ratio $\frac{\text{Decrease in Formol N}}{\text{Decrease in Total N}}$ in Other Experiments.

Time.	Mash D.	Mash E.	Mash F.
<i>hrs.</i>			
2	0.14		
4	0.32		
6	0.55		
8	0.51	0.66	
10	0.44		
12	0.57		
14	0.48		
16	0.53	0.83	0.45
18	0.55		
20	0.56		
22	0.57		
24	0.53		

marked decrease in the amount of ammonium salts used by us here will diminish both yield and nitrogen content of the resulting yeast. The quantity of non-formol nitrogen utilized, however, remains relatively the same part of the total throughout. In these several experiments it does not lead to a constant final value of non-formol nitrogen, nor does the amount used depend upon the relative proportions of formol and non-formol nitrogen present, for these vary widely during the same experiment. If a sort of digestion of the nitrogenous bodies of the beet molasses took place, this should depend to some extent on the quantity of yeast present, and therefore, be larger toward the end of the

mash. This would lead to an increased use of non-formol nitrogen at this time, which is not observed.

It looks as though our ideas of the unfitness of molasses nitrogen for yeast growth will have to be modified, and that the more complex nitrogen bodies of the molasses furnish a form of nitrogen which, while not absolutely necessary to yeast growth, is certainly chosen by the organism.

In any case it is a fact that nitrogen from the beet sources is used parallel to nitrogen from the ammonium salts, and in such a way that a rough proportionality exists between the two throughout the growth of the yeast. That the beet nitrogen should be used seems very plausible; but we lack any explanation of the observed proportionality.

SUMMARY.

We have attempted the collection of data regarding the changes occurring in the rapidly growing culture of *Saccharomyces cerevisiæ*. The medium of growth was one consisting of beet sugar molasses and ammonium salts. The mash was thoroughly aerated and treated as far as possible in the manner which empirical observation has shown in the industry to give the maximum yeast crop of well nourished yeast, and the experiments were continued beyond the point of growth cessation. Various determinations made at close intervals throughout the experiments enable the construction of time-concentration curves of the constituents under observation. Such curves give a picture of the rate of variation in these constituents during the period of yeast growth, rather than a summary of the final outcome of the fermentation. A method for determining the hydrogen ion concentration of liquid cultures is described; and also a method for determining yeast crops.

As soon as yeast is placed in the molasses solution, a rapid inversion of sucrose commences. There begins at once a loss of total sugar from the medium, a corresponding loss of total solid matter, and an appearance of carbon dioxide and alcohol. These changes take place most rapidly during the early hours of the experiment. At the end of 8 hours, the disappearance of sugar and total fermentable solids is practically complete. The alcohol concentration is at its maximum at this time, as is also

the hydrogen ion concentration of the liquid. Alcohol disappears gradually thereafter, partly no doubt due to aeration, and partly we believe by conversion to some other compound. With this gradual loss of alcohol there is a slow but steady production of carbon dioxide, although no further loss of sugar to account for it.

A very considerable increase in the weight of the yeast occurs during the experiment. The logarithmic law of yeast multiplication was found to hold when this weight was measured instead of the cell count. The weight continues to increase for a long time after all the sugar has been removed from the liquid, but in the absence of sugar this increase is not as rapid. It also proceeds logarithmically, however, with a velocity constant roughly one-tenth that of the prior rapid logarithmic phase. The addition of more sugar after the first supply has been exhausted produces a farther spurt of yeast growth, together with all the usually attendant changes. The spent liquor of the mash is not appreciably toxic to new growths of yeast. Sugar is not necessary for yeast growth, but the speed of growth is greatly increased by its presence.

During the disappearance of sugars, a small quantity of unassimilable reducing substance is formed in the solution. The disappearance of sugar roughly conforms to the mechanical conception of Sclator regarding the fermentation of sugar by yeast. By comparing the rate at which the sugar disappears with the rate at which it is inverted, the former is seen to be directly dependent on the velocity of yeast growth and the time, while the latter is not. It can be argued that this furnished additional evidence of the extracellular nature of the hydrolyzing enzymes in the case of healthy and growing yeast.

Changes in the nitrogen concentration differ from changes in sugar concentration and in general parallel the increase in weight of the yeast throughout the entire time of the experiment, indicating that nitrogen is used merely as the yeast grows. There is no nitrogen-sparing effect of carbohydrates. The nitrogen is not derived from the atmosphere, but comes both from the beet molasses and from the ammonium salts present; both forms are used in a fairly definite proportion which remains reasonably constant throughout the experiment, independent of the variations in amounts of either.

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STUDIES IN YEAST METABOLISM. II.

CARBON DIOXIDE AND ALCOHOL.

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In the preceding paper we have recounted some experiments on the growth of baker's yeast in a heavily aerated wort of beet molasses containing ammonium salts as an added source of nitrogen and phosphate as an additional food and buffer material. Some of the chemical changes occurring in the medium during the growth of the yeast were studied, and the data presented and discussed.

During our first experiments alcohol determinations were made, showing a maximum at the time of approximate sugar disappearance, also coincident with a change in the velocity of yeast growth to roughly one-tenth its former value. Evidently considerable alcohol was being volatilized by the air current passed through the medium, so in order to study farther the production of alcohol, it was necessary to determine accurately the amount carried out of the system by the air. The carbon dioxide produced would naturally be determined in a similar way. The object of this paper is to record a second series of experiments made upon the same media and with the same yeast used previously, but under conditions allowing such determinations of alcohol and carbon dioxide to be made.

In the first series of experiments one attempt only was made to determine the amount of carbon dioxide produced (Mash H). This experiment was made in a closed vessel from which the issuing air was conducted through large gas-washing bottles containing a potassium hydroxide solution. It was impossible to pass as rapid a stream of air through this system as through our other mashes, and subsequent experience also showed that our form of

absorption apparatus was not sufficiently efficient to retain all the carbon dioxide. The results, however, are included in the data of our first paper for the sake of the other determinations which were made simultaneously; and the carbon dioxide found to be evolved is plotted here against time because, while the results are low, they are low by a small and presumably an approximately constant amount, and the form of the curve so obtained is interesting.

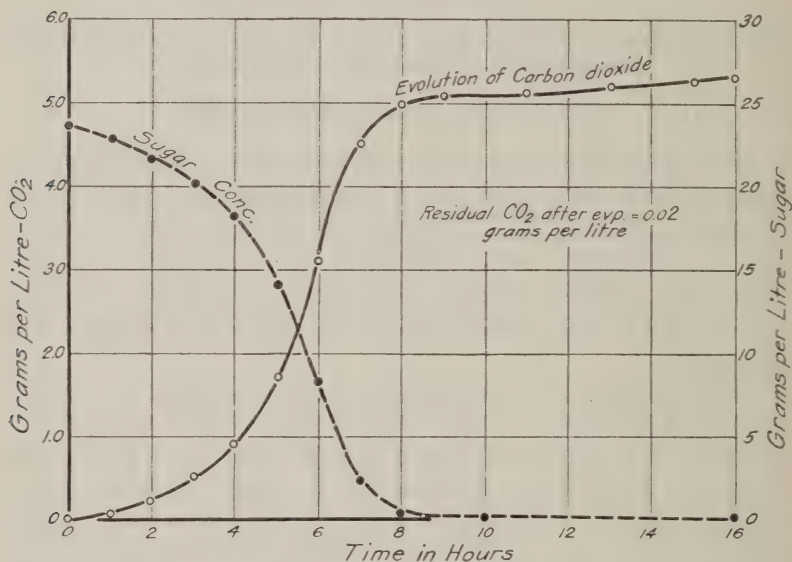


FIG. 1. Carbon dioxide evolved-sugar concentration.

From this curve (Fig. 1) it is seen that the greater part of carbon dioxide is evolved coincident with sugar decomposition. But it is significant that after the removal of all the sugar, carbon dioxide continues to come off.

Apparatus and Methods.

To remove carbon dioxide and alcohol from the air issuing from the yeast culture, and at the same time not to reduce the aeration below that used in our previous experiments, the work was performed on a smaller scale. Usually about three-quarters of a

liter of medium¹ was placed in a tall cylindrical jar, immersed in a thermostat. On passing air through a rose-tipped aerating tube in the jar at the rate of 5 liters per minute, about the same aeration was produced as in our earlier work. The complete apparatus as eventually used is sketched in Fig. 2.

A and B are washing bottles containing 50 per cent potassium hydroxide solution, or moist soda-lime, to remove atmospheric carbon dioxide. A test of this point with the apparatus as finally set up showed that after 8 hours aeration at 8 liters per minute, the carbon dioxide not removed from the air was less than our carbon dioxide method was capable of measuring.

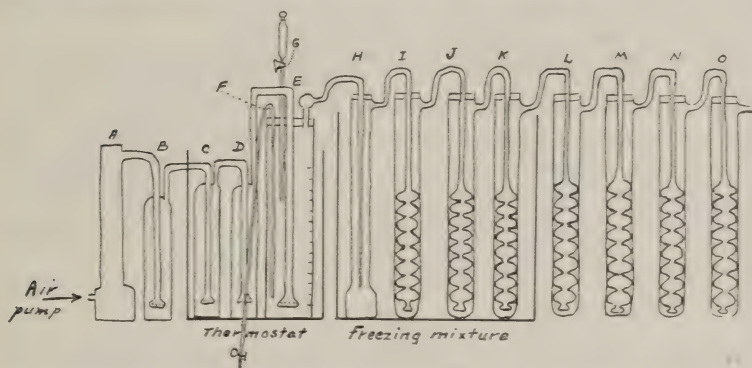


FIG. 2. Apparatus used for the estimation of alcohol and CO_2 carried from the mash by aeration.

C and D are gas-washing bottles containing water. They are immersed in the thermostat in order to saturate the air with moisture at the temperature of the experiment. By this means practically no evaporation of the medium contained in E was observed. Since the air was furnished by a pressure pump, the siphon tube F could be used for removing samples from E by opening a pinch-cock, and the inlet G (separatory funnel with a firmly fitting stopper) could be used to introduce liquid material into the mash while

¹Medium composed as follows:

	gm. per l.
Beet molasses.....	48.0
Ammonium dihydrogen phosphate.....	1.2
“ sulfate.....	1.6

the latter remained under pressure. A few drops of olive oil to prevent foaming were always added in this way.

H is an empty tube, *I*, *J*, and *K* are washing bottles containing calcium chloride solution, and *L*, *M*, *N*, and *O* are similar bottles containing 50 per cent potassium hydroxide solution. The former set is for the removal of alcohol, the latter for the carbon dioxide.

The bottles are about 30 inches tall and 3 inches in diameter, and of the shape shown in Fig. 2. The inside tube on which the bulbs are blown is perforated at the bottom with a number of fine holes, similar to a rose top burner, which distribute the air equally around the periphery of the bottle. The top is closed with a rubber stopper. This type of absorption apparatus we found to be the most efficient for our purpose.

Since the head in this apparatus is considerable, an initial pressure of at least 4 pounds per square inch was required in order to pass the desired quantity of air through the apparatus. This amount was gauged at frequent intervals (rather inaccurately) by connecting a large rubber tube to the outlet of *O*, and collecting the air in an inverted 4 liter cylinder, initially filled with water, the time required to fill the cylinder being measured.

Determination of Alcohol.

The mash liquor was analyzed for alcohol by distilling 50 or 100 cc. samples over a little chalk, collecting half the volume of distillate, and determining the specific gravity in a 25 or 50 cc. gravity bottle at temperatures very near 60°F., making the customary corrections.

The complete removal of alcohol vapor from the air, however, was quite a difficult task, and was finally accomplished by passing the air through the succession of washing bottles shown, each containing 200 to 300 cc. of cold calcium chloride solution (about 30 per cent). This solution was first saturated with a current of carbon dioxide, then thoroughly aerated. If a stronger solution is used, a deposit of crystals obstructs the air current. The washing bottles containing the solution were immersed in a bath of cracked ice and salt or hydrochloric acid, which should have a temperature about -15°C . Practically all the moisture in the air is removed, which tends to dilute the solution in the bottles, and to freeze on the inside of the aerating tubes, often closing them.

It was found very helpful to pass the air first through a jet into an empty tube, also chilled.

When it was desired to determine the alcohol, another set of bottles was quickly substituted in the train to continue the run. The contents (including the condensed water of the empty tube) were distilled with chalk until two-thirds the original volume of the liquid was obtained. If the distilling flask went dry before this, a little water was added after cooling, and the distillation continued. Noyes (1) has mentioned that alcohol may be quantitatively distilled from strong solutions of calcium chloride. The gravity of the distillate was then taken in the customary manner (2).

TABLE I.
Alcohol Recovered in a Series of Test Experiments.

Total alcohol at start.	Concentration of alcohol in liquid at start.	Time of aeration.	Air.	Alcohol recovered.						
				Original solution.	Tube I.	Tube II.	Tube III.	Tube IV.	Total.	Error.
gm.	gm. per l.	hrs.	l. per min.	gm.	gm.	gm.	gm.	gm.	gm.	per cent
14.30	29.6	2	2-3	10.70	3.22	0.13			14.05	-1.8
4.85	9.7	5	5-6	2.25	2.12	0.47	0.03	0.00	4.87	+0.4
8.24	16.4	3½	4	5.48	2.58	0.20	0.00		8.26	+0.2
12.87	25.7	5	6-7	7.40	4.60	0.68	0.08		12.76	+0.8

In order to test this method, a known amount of dilute alcohol was placed in the jar ordinarily occupied by the yeast culture, and was aerated for several hours, at a temperature close to 28°C. The amount of alcohol remaining in the original solution as well as that found in each of the calcium chloride tubes were then determined. The results are tabulated in Table I.

From our experience here three calcium chloride bottles and one empty tube for condensing the water seem adequate to remove all the alcohol, and the final apparatus was arranged accordingly.

Determination of the Carbon Dioxide.

The chief difficulty was in getting complete absorption from the large volumes of air. This led to the adoption of the type of washing bottle shown. No carbon dioxide was absorbed by the last bottle.

We chose to determine the carbon dioxide by titration instead of with soda-lime tubes. While not so accurate as the gravimetric procedure, this has the advantage of being freer from the suspicion of determining other substances as carbon dioxide.

The titration of the carbonate formed in the absorption bulbs was done using the well known method of neutralizing the diluted liquid first to phenolphthalein and next to methyl orange. This is not an easy titration; it cannot be done by artificial light, and needs a very considerable degree of experience. The details of the method are of importance. The strong potassium hydroxide liquor, from one or more of the bulbs, was diluted to a definite volume, generally about three times the original. An aliquot of this stock solution (usually one-fifth) was placed in a beaker together with an equal amount of cracked ice. A solution of HCl, about 10 per cent, not standardized, was then run in from a burette, very carefully, and with adequate but quiet stirring. The tip of the burette must dip to the bottom of the beaker. After considerable acid has been added, phenolphthalein is introduced and the neutralization carried on with the strong acid until it is almost complete. The indicator loses its rosy tint by this time, and becomes pink. The nearly neutralized liquid was then placed under a similar burette containing the standard acid, approximately half normal, and the neutralization to phenolphthalein finished. After this the methyl orange titration is made in the usual manner, and represents the carbon dioxide to be determined.

By using the strong acid at the beginning, it is possible to take much larger aliquots. Containing, as the solutions do, so much hydroxide compared with carbonate, the customary dilute acid would inconveniently increase the volume of the solution. No potassium chloride was added to the liquid to repress the ionization of H_2CO_3 , since considerable is formed in the preliminary titration.

Careful blanks must be performed on all solutions of potassium hydroxide, and titrations should be made in triplicate at least. Even then the method is none too accurate, as the following test results show (Table II). Here pure sodium carbonate in known amounts was added to solutions of potassium hydroxide of strength comparable to those used in our work, and the carbonate determined by the foregoing method.

The results are those of six consecutive determinations, and show the necessity of doing several titrations for each determination. With these precautions we think the method gives values about 5 per cent low.

The results recorded here are not corrected to represent this probable error, since the differences dealt with in this paper are much larger and also in the opposite direction; that is, we find more carbon dioxide than theoretical considerations would lead us to expect.

TABLE II.
Showing Accuracy of the Carbonate Titration.

No.	Carbon dioxide added as Na_2CO_3 .	Carbon dioxide found by titration.	Error.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
1	0.1175	0.1144	-2.6
2	0.2350	0.2156	-8.5
3	0.2350	0.2200	-6.4
4	0.4700	0.4675	-0.5
5	0.4700	0.4460	-5.1
6	0.9400	0.9000	-4.3

Data.

Several experiments were now run, using our beet molasses-ammonium salt medium and the methods just described. Since the sugar has been shown to last only for 7 or 8 hours under these conditions, the greater part of our attention was confined to the first 8 hours of the experimental time.

Data summarizing the findings are shown in Table III.

The temperature of these experiments was $28 \pm 0.2^\circ$. Aeration was at the rate of 6 to 8 liters per minute. The carbon dioxide recorded at each time is the total amount volatilized from the mash up to that hour. The gas does not collect in the liquid when so much air is passing through. The figures for alcohol represent the alcohol concentration existing in the yeast culture at the time of measurement plus all the alcohol previously volatilized from the mash. The actual concentration of alcohol in the mash itself is much less, particularly in the latter part of each run. For example, in Run III, which is the most complete, the alcohol figures in detail were as given in Table IV.

From these results the quantity of carbon dioxide formed is greater than that equivalent to the alcohol. This leads to values

TABLE III.

Showing the Alcohol and Carbon Dioxide Liberated by Growing Yeast.

Run No.	Time.	Sugar used calculated as invert sugar.	Yeast increases.	CO ₂ liberated.	Alcohol.
	<i>hrs.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>
I	0				
	3	3.32	0.88	1.45	1.51
	6	17.02	3.86	7.19	5.98
	8	23.34	5.48	11.39	8.02
II	0				
	3	5.52	1.81	2.82	2.08
	6	22.39	4.89	11.34	7.51
	8	23.76	6.35	13.53	10.14
	24		8.85	14.78	5.17
III	0				
	3	7.08	1.65	3.07	2.71
	6	17.36	3.97	8.83	6.27
	8	17.69	4.33	9.98	6.00
	16½	17.96	5.93	12.58	5.00
	28		6.73	12.96	4.83

TABLE IV.

Alcohol from Run III.

Time.	Alcohol found in the liquid.	Alcohol accumulated in the absorption bulbs.		Total alcohol at time of measurement (last column of Table III).	
		Time.	Amount.	Time.	Amount.
<i>hrs.</i>	<i>gm. per l.</i>	<i>hrs.</i>	<i>gm. per l.</i>	<i>hrs.</i>	<i>gm. per l.</i>
3	2.40	0 - 3	0.31	3	2.71
6	4.60	3 - 6	1.36	6	6.27
8	3.20	6 - 8	1.13	8	6.00
16½	0.80	8 - 16½	1.40	16½	5.00
28	0.00	16½ - 28	0.63	28	4.83

for the $\frac{\text{alcohol}}{\text{CO}_2}$ ratio which are not only not the generally accepted ratio, but are not constant among themselves (Table V).

The disappearance of alcohol noticed in the experiments done in open vessels was then attributed to volatilization. This explanation seems now untenable, and an actual consumption of alcohol must be assumed to explain the facts. The ratio of alcohol to carbon dioxide has been carefully determined by quite a number of workers (3), and the values found by them were in no case very far away from that predicted by the old equation of Gay-Lussac:

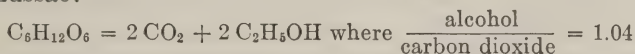


TABLE V.
Value of the $\frac{\text{Alcohol}}{\text{CO}_2}$ Ratio.

Run No.	Time.	$\frac{\text{Alcohol}}{\text{CO}_2}$
	<i>hrs.</i>	
I	3	1.04
	6	0.83
	8	0.70
II	3	0.74
	6	0.66
	8	0.75
	24	0.35
III	3	0.88
	6	0.71
	8	0.60
	16½	0.40
	28	0.37

It must be remembered, however, that the conditions of our yeast growth are materially different from those employed by any of these other men.

The difference lies chiefly in the aeration of our solutions and the resultant increase in yeast growth. As an illustration of this, the same yeast and media were allowed to react at the same temperature, but without any air except that above the liquid in the flask, and a slow aeration for 2 or 3 minutes every 2 hours in order to stir the liquid and remove most of the carbon dioxide, the accumulation of which in the liquid was undesirable from the

standpoint of comparison with our aerated growths. Unfortunately for our alcohol-CO₂ ratios, the carbon dioxide was not completely removed by these means until the end of the experiment when a more vigorous air current was used. The difference, however, between these unaerated mashes and those just recorded, is quite striking.

Owing to the comparative slowness of fermentation, the time required for these experiments was correspondingly increased (Table VI).

The influence of air on alcoholic fermentation and yeast growth was noted by Pasteur (4), who recognized that larger growths occurred when the exposed surface of a beer wort was large, but that less fermentation took place. Pasteur also held that the

TABLE VI.
Alcohol and Carbon Dioxide from Non-Aerated Yeast Growth.

Run No.	Time.	Sugar used.	Yeast increase.	Carbon dioxide.	Alcohol.	$\frac{\text{Alcohol}}{\text{CO}_2}$
	<i>hrs.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	
I	8	19.01	2.40	6.58	8.00	1.21
II	5½	2.3	0.27	0.82	0.80	0.98
	9¾	12.0	1.21	3.83	4.35	1.12
	20½	20.7		6.03	8.05	1.33
	48½	22.2		7.03	8.15	1.16

budding of yeast was aided by free oxygen. The influence of oxygen on the growth rate of yeast has been in more recent times accurately demonstrated by H. T. Brown (5). Schützenberger (6) showed in modern terminology that yeast was able to oxidize its cell content, giving off carbon dioxide at the expense of atmospheric oxygen, and Prior (7), some years later, distinguishes yeast growth and fermentation as two entirely separate processes. The recent work of Neuberg, to which no specific reference is needed, enables us now to regard fermentation as a chemical process. The passage of air and other gases through growing yeast cultures was also investigated by A. J. Brown (8) who found the fermentation accelerated, as well as the growth, but Brown did not continue his experiments to the point of exhausting the sugar supply. Korff (9) made a very elaborate study of the effect of

air, carbon dioxide, and hydrogen upon the chemical composition of the culture medium. Korff's aeration was evidently not very rapid, for during the 14 days duration of his experiments, the sugar was not completely exhausted, nor was any decrease in the alcohol noted. He concludes, however, that the two functions, growth and sugar fermentation, are distinct, and vary independently in different species of yeasts, a fact which the technical worker is just beginning to realize, though in Korff's time, yeast makers were blowing air into their tubs to increase the yields.

Giltay and Aberson (10), working with sugar solutions, concluded that with strong aeration only 75 per cent of the sugar was decomposed according to the equation of Gay-Lussac, while without air 90 per cent was so broken up.

The results of our experiments do not seem to be in discord with those of previous workers, but as far as we are aware, the extent to which the changes can take place that are brought about by aeration, has not been hitherto noted.

The continued increase in carbon dioxide was occurring very probably at the expense of the alcohol, either by passage of the carbon through the yeast cell or by direct oxidation, and this led us next to investigate the behavior of mixtures of dilute alcohol and yeast under these same conditions. Accordingly, solutions containing 2 to 3 gm. of yeast (calculated as dry substance) and 10 to 12 gm. of alcohol per liter, with ammonium phosphate and ammonium sulfate (1.2 gm. of $\text{NH}_4\text{H}_2\text{PO}_4$ and 1.6 gm. of $(\text{NH}_4)_2\text{SO}_4$ per liter) were aerated for periods of approximately 7 hours, the air being first freed from carbon dioxide and then saturated with moisture at the temperature of the experiments (28–29°). In these experiments a decrease in the quantity of alcohol, a production of carbon dioxide, and a very slight increase in the weight of the yeast were constantly observed (Table VII).

It was also necessary to find out the behavior of the same yeast with a similar concentration of alcohol but in absence of air, and without alcohol in the presence of air. In neither case did any considerable action take place. The yeast lost slightly in weight instead of gaining, and a much smaller amount of carbon dioxide was evolved.

In the non-aerated control experiment (Table VIII), the yeast-alcohol mixture was stirred in a flask fitted with a mercury seal.

No attempt was made to remove the air contained in the flask. The flask was attached to the usual absorption apparatus, and at the end of the experiment a brisk current of air passed through for a few minutes to remove any carbon dioxide. In the control from which alcohol was omitted, the experiment was conducted in the same way as described for the alcohol-yeast mixtures.

TABLE VII.
Aeration of Alcohol-Yeast-Ammonium Salt Mixtures.

Run No.	I	II	III	IV
Yeast at start, gm. per l.	2.80	3.70	2.04	3.71
“ “ end, gm. per l.	3.60	3.77	2.58	
“ increase, gm. per l.	0.80	0.07	0.52	
Alcohol at start, gm. per l.	9.68*	12.87	9.68	12.87
“ “ end, gm. per l.	8.21	8.05		10.00
“ decrease, gm. per l.	1.47	4.82		2.87
Carbon dioxide evolved, gm. per l. .	0.82	0.87	1.19	0.57

TABLE VIII.
Control Experiments on Alcohol-Yeast-Air Mashcs.

	Non-aerated.	No alcohol.
Yeast at start, gm. per l.	3.38	3.48
“ “ end, gm. per l.	3.24	3.16
“ decrease, gm. per l.	0.14	0.22
Alcohol at start, gm. per l.	9.68	0.00
“ “ end, gm. per l.	9.50	0.00
“ decrease, gm. per l.	0.18	
Carbon dioxide evolved, gm. per l.	0.00	0.15

In view of these experiments we feel that the actual loss of alcohol observed in our previous work, and the excessive production of carbon dioxide, are in part at least explained by the transformation of alcohol into carbon dioxide by yeast in the presence of air. The carbon dioxide found is by no means the equivalent of the alcohol used up, so that we believe other products, which we have

not yet detected, are formed simultaneously. Whether the alcohol suffers a direct oxidation in the presence of the yeast, or whether a passage of the alcohol carbon through the cell materials takes place, resulting in its ultimate appearance as carbon dioxide, are questions for which these data offer no solution. There is, however, some historical background for assuming such an oxidation. Kruis and Rayman (11) report finding the alcohol in old and exposed beers to have been largely oxidized to carbon dioxide and water, and Trillat and Sauton (12) have shown that acetaldehyde was produced from alcohol by the action of yeast and air together.²

The increase in yeast weight observed by us is only slight, and no signs of budding were seen in the microscope. Stained with iodine, the cells after aeration in alcohol seemed to contain more glycogen and to be uniformly better developed. The increase in weight of the yeast appears, therefore, more probably to be due simply to a "fattening" of the cells, rather than to their multiplication. Lindner and Cziser (13) and again Stockhausen (14) have reported the assimilation of alcohol by yeasts, with attendant multiplication, although this was small and slow. We are inclined to think that in our experiments the effect of the alcohol as a source of carbon for growth was very slight; but it is certain that aeration in the alcohol solutions produced a yeast which microscopically had every appearance of being better developed.

Abderhalden (15) has recently discovered that in the presence of animal charcoal and yeast, alcohol is oxidized to acetaldehyde and also to carbon dioxide. This he attributes to an enzymic mechanism, and not to any metabolism, strictly speaking, of the carbon by the cells. We are inclined to regard the disappearance of the alcohol as a similar phenomenon.

²Similar findings regarding the production of carbon dioxide from alcohol under aerobic conditions have been recently announced by Lundin (Lundin, H., *Biochem. Z.*, 1923, cxli, 342; cxlii, 454).

This author, however, obtained relatively considerable increase in the weight of yeast present, which is attributed to alcohol assimilation and carbohydrate formation by the cell. The carbon dioxide is regarded as a product of cell respiration. From this view-point, which was first developed for analogous systems containing sugars, the carbon balances have been carefully worked out.

SUMMARY.

The production of carbon dioxide and of alcohol by an aerated yeast culture has been studied by methods which permit the measurement of the alcohol as well as the carbon dioxide removed in the air current. The production of alcohol is found to be much less than that required by the usual fermentation equation, and the amount of alcohol to decrease gradually after the sugar is all decomposed until finally the liquid contains none. A definite destruction of the alcohol in the solution is shown. On the other hand, the amount of carbon dioxide obtained is greater than would be predicted on the basis of sugar removal. It continues after the sugar is entirely gone. In similar experiments made on alcohol solutions and yeast, the alcohol likewise disappears, carbon dioxide is formed, and the yeast gains weight slightly. In the absence of air these findings are not made. The opinion is advanced that the removal of alcohol is an oxidation which does not involve metabolism of the carbon, that products other than carbon dioxide are formed by this means, and that the increase in yeast weight is due to a development of the cells rather than to their multiplication.

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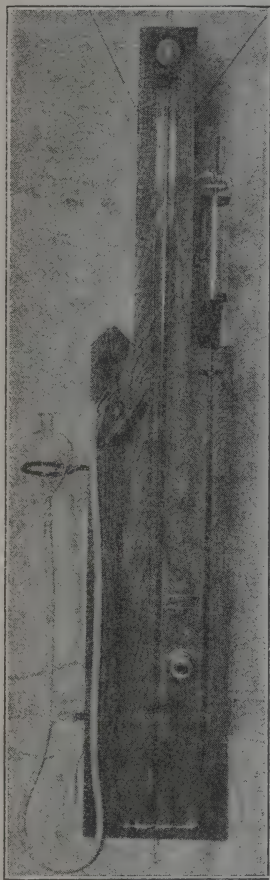
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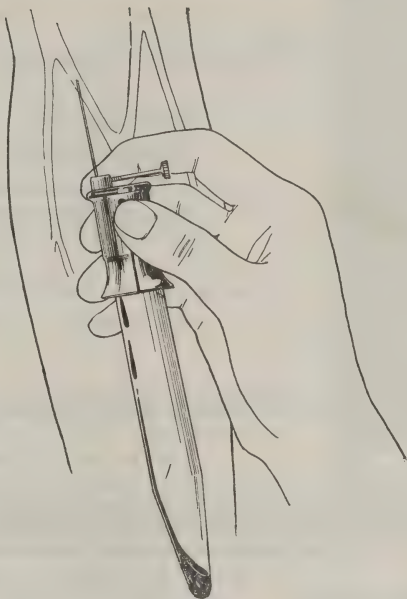
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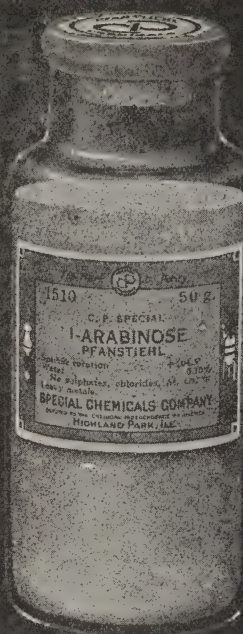
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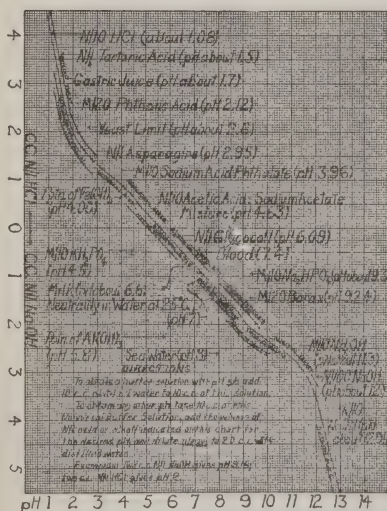
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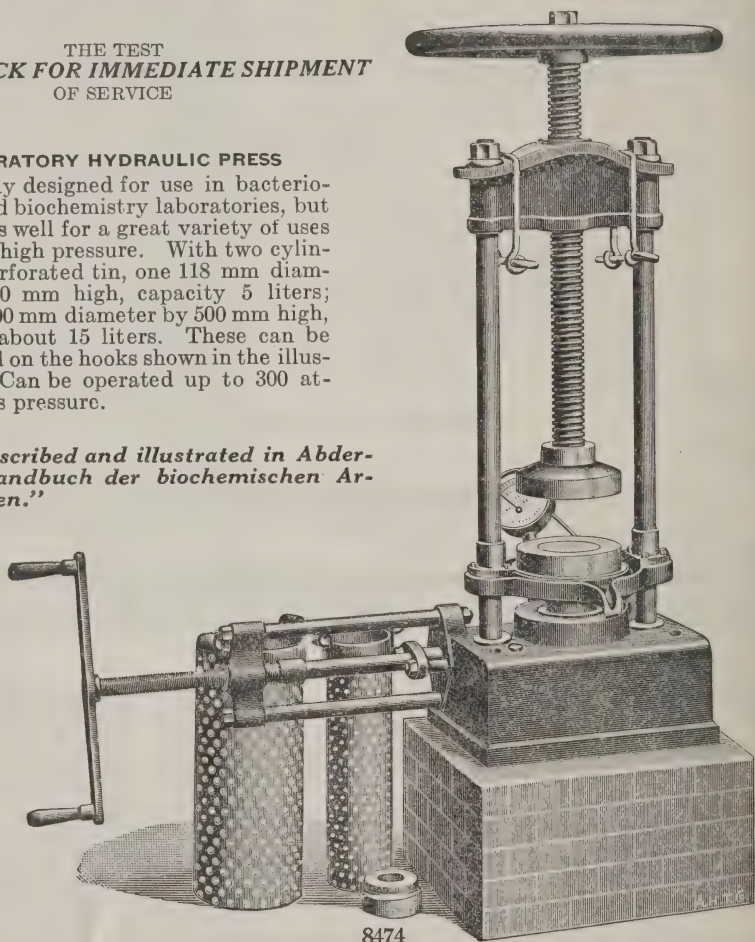
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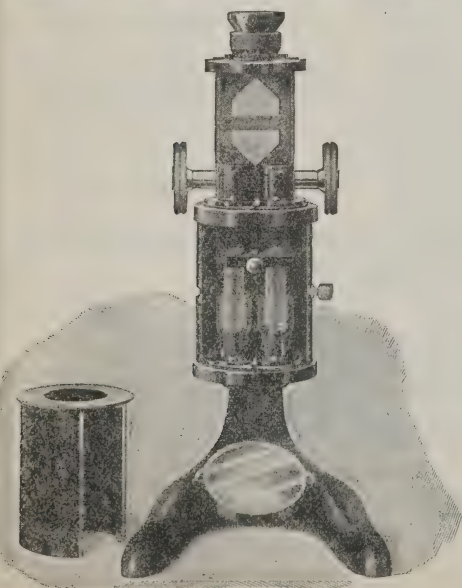
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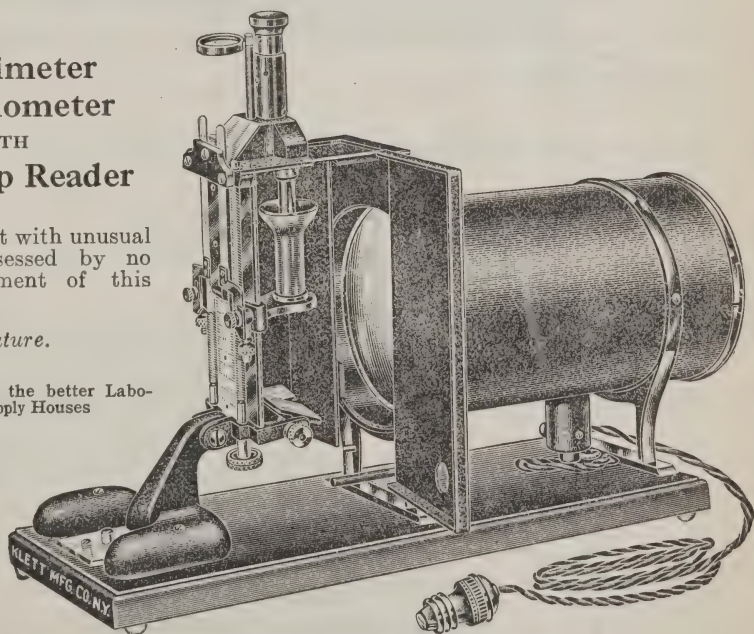
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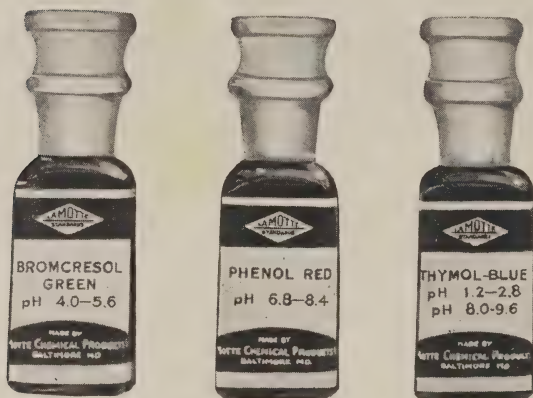
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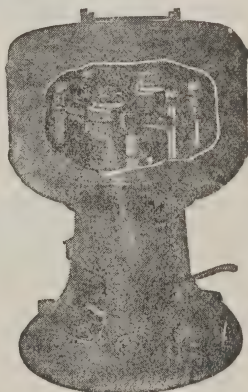
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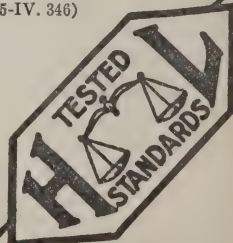
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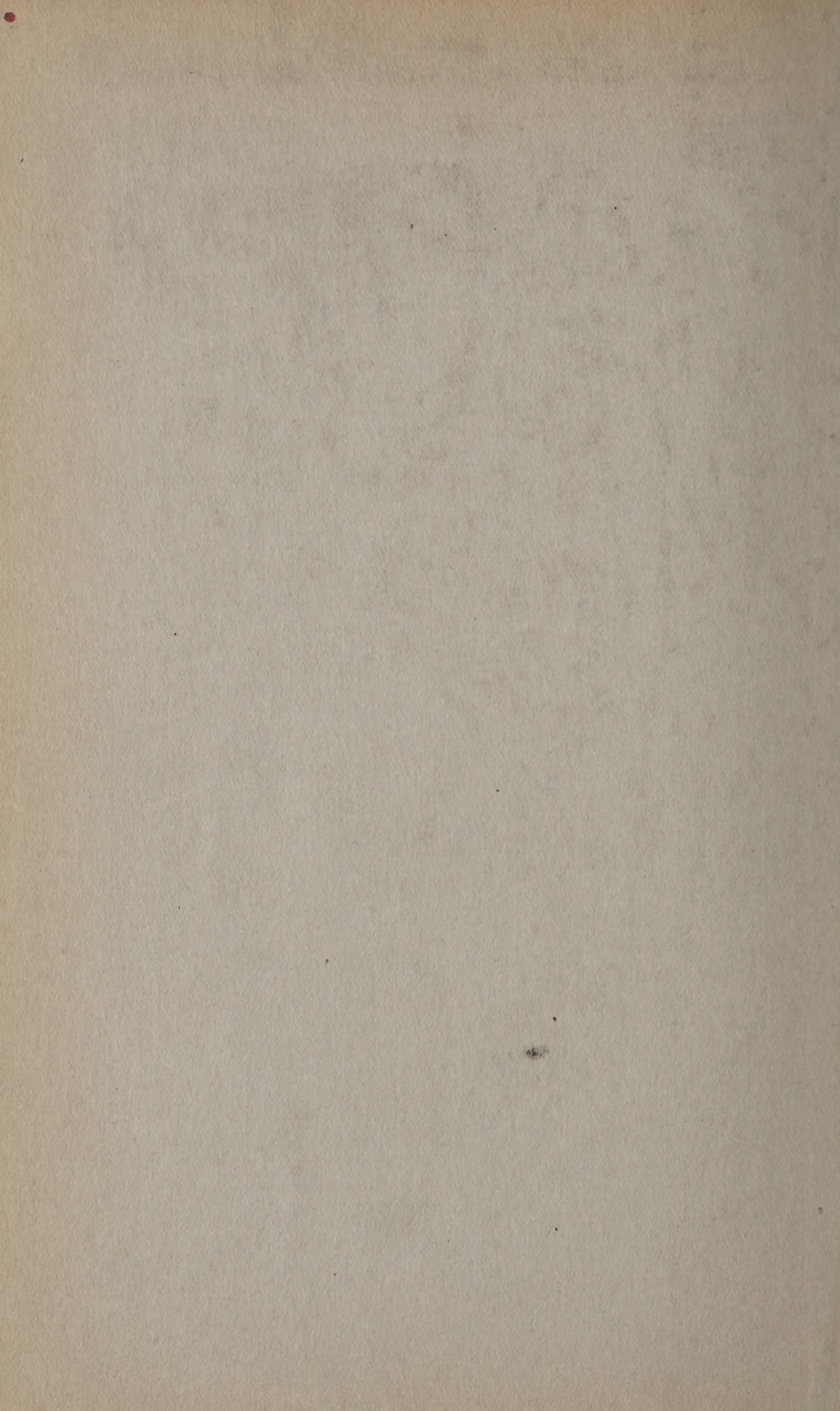
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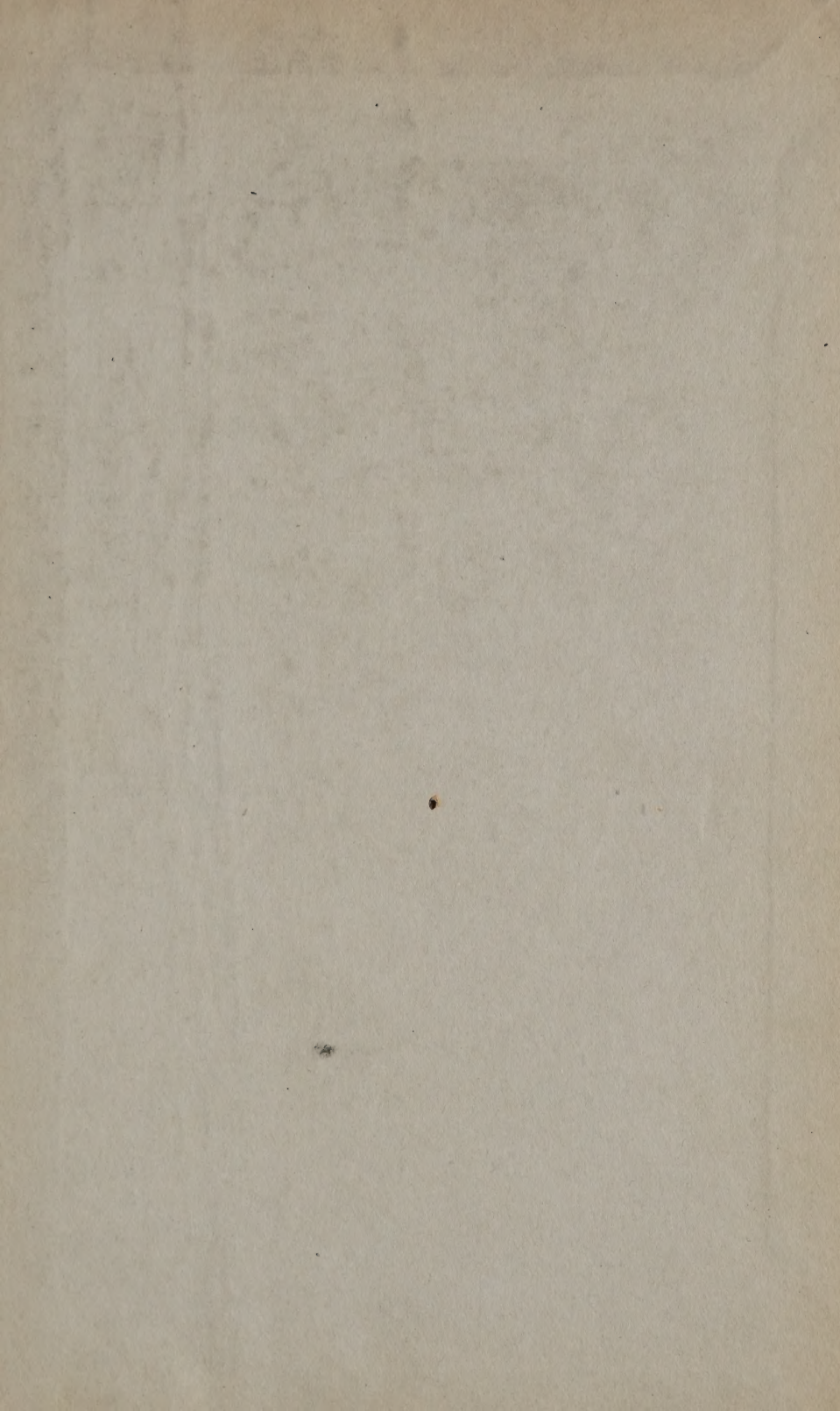
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